CHAPTER 3

The chemistry of soil organic colloids

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3.1 INTRODUCTION

The complete soil organic fraction is made up of live organisms, and their undecomposed, partly decomposed and completely transformed remains. Soil organic matter is the term used to refer more specifically to the non-living components which are a heterogeneous mixture composed largely of products resulting from microbial and chemical transformations of organic debris. These transformations, known collectively as the humification process, give rise to humus, a mixture of substances which have a degree of resistance to further microbial attack.

It is well known that soil organic matter has a beneficial effect on soil conditions and fertility. Amongst the more important functions which have been attributed to it are:

(i) the formation and maintenance of good soil structure;
(ii) the improvement of water entry into, and retention by the soil;
(iii) the retention of plant nutrients as the result of its cation-exchange properties;
(iv) the release of nitrogen, sulphur, phosphorous and possibly some trace elements as the result of degradation and mineralization;
(v) increasing soil temperature by enhancing absorption of solar radiation due to its dark colour.

It is probable that most of these beneficial effects can be attributed to the humus fraction of soil organic matter. The synthesis and degradation of humus is a dynamic process which attains an equilibrium in a particular soil environment.

This equilibrium can be disturbed by environmental changes, such as variations in soil water levels, alterations in cultivation practices, etc. Substantial depletion of the organic matter content of a fertile soil can lead to the deterioration of the soil structure and the loss of fertility.

Since soil organic matter is a heterogeneous mixture it is necessary for purposes of discussion to subdivide it into groups which have similar morphological or chemical characteristics. Here we are adopting a classification based largely on proposals by Kononova (1966, 1975). In this system soil organic matter is separated into two major groups:

(I) Unaltered materials, which include fresh debris and non-transformed components of older debris;
(II) Transformed products, or humus, bearing no morphological resemblance to the structures from which they were derived.

These transformed components are often referred to as humified products, but in fact they consist of both humic and non-humic substances and can be therefore subdivided as follows:

(IIa) Amorphous, polymeric, brown coloured humic substances which are differentiated on the basis of solubility properties (see Section 3.3.1) into humic acids, fulvic acids, and humins;
(IIb) Compounds belonging to recognizable classes, such as polysaccharides, polypeptides, altered lignins, etc. These can be synthesized by microorganisms or can arise from modifications of similar compounds in the original debris.

Humic substances are widely distributed in soils and waters, and are probably the most abundant of the naturally occurring organic polymers. Reserves of soil humic acids (the most abundant of the humic substances), for instance, are estimated to be of the order of $2–3 \times 10^{12}$ tonnes (Bazilevich, 1974). Yet their structures and properties are not sufficiently well understood. However, significant advances can soon be expected in these areas because of recent progress in degradation techniques and in instrumentation.

In order to carry out meaningful investigations of their compositions it is important to be able to extract humic materials in representative yields from soil; to separate these from co-extracted soil minerals and from non-humified substances; to isolate the relevant gross fractions; and to fractionate these further into components which are reasonably homogeneous with regard to molecular sizes and charge densities. If this can be successfully achieved results from chemical and physical measurements will have greater significance.

It is convenient to describe polymers in terms of primary, secondary, and tertiary structures. Primary structures can be considered to be the monomer units or 'polymer building blocks' which are released into the digest during degradation reactions. Secondary structures define the sequential arrangements of these units in the polymer, and tertiary structures describe the sizes and

three-dimensional shapes of the polymers. When the primary structures are known it is possible to distinguish the chemical units which govern the reactivities of the structures. Detailed knowledge of the secondary and tertiary structures would allow predictions of polymer behaviour in different media, and would be especially valuable for a more complete understanding of interactions with soil inorganic colloids, sorbed organic molecules, and inorganic ions. Thus, advances in structural studies can provide essential information to help resolve many questions such as the stabilisation of soil structure, and the binding of pesticides and of heavy metals in the soil environment.

Some aspects of the role of humic substances in soil are discussed in the companion volume and by Kononova (1966, 1975), and Schnitzer and Khan (1972). This chapter deals in part with the composition and properties of soil polysaccharides (Section 3.6), but is primarily concerned with the chemical and physical properties of humic substances. We begin with a discussion of the isolation and purification procedures which are a necessary preliminary to the determination of composition, structure, shape, size and charge properties. When dealing with these subjects some emphasis is placed on the scientific principles on which the techniques are based and on the interpretation of the results obtained.

3.2 EXTRACTION OF HUMIC SUBSTANCES

Ideal solvent systems would quantitatively isolate homogeneous components of humic substances from the soil. Such systems are not known, and are unlikely to be devised because of the inevitable overlap of solubility properties among the vast numbers of organic materials which are present in the soil environment. For that reason most effort has been concentrated on finding solvents that will extract high yields of humic materials without significantly altering the compositions of the natural polymers. Large scale extractions are often preceded by preliminary treatments with organic solvents, such as benzene and ether, in order to remove waxy materials. Such solvents inevitably remove many of the low molecular weight organic compounds which generally compose only a small fraction of the soil organic matter.

We shall be concerned here with procedures used to extract the brown humic polyelectrolytes. At the pH values of most fertile soils these polyelectrolytes are rendered insoluble through ion-exchange reactions with polyvalent metal cations. Thus soils are usually pretreated with mineral acid which removes metallic cations, helps to break links between humic materials and sesquioxides, and dissolves soil carbonates. The carboxyl and phenolic hydroxyl groups in the resulting H^+-ion saturated humic materials are not dissociated, and this allows the polymers to associate through hydrogen bonding and van der Waals forces mechanisms. Thus the extraction of H^+-humic substances can be regarded as a problem of polymer solubility. More commonly, however, these

substances are dissolved by replacing the H^+ with solubilizing ions such as Na^+, K^+, or NH_4^+, by using neutral or basic solutions of the cations.

Whitehead and Tinsley (1964) have outlined the criteria for effective solvents for humic substances. These are:

(i) a high polarity and a high dielectric constant to assist the dispersion of the charged humic molecules;
(ii) a small molecular size to penetrate through the humus polymers;
(iii) the ability to disrupt the existing hydrogen bonds, and provide alternative groups to form humic–solvent hydrogen bonds;
(iv) the ability to immobilize metallic cations.

From the treatment which follows it will be seen that solvents or solvent systems which give good yields of humic products satisfy most or all of these criteria.

3.2.1 Extraction of Ionized Humic Substances

Polymers are dissolved in two steps. First solvation, or a swelling process takes place to form a gel which results from the absorption of solvent molecules by the surface causing the polymer to change its average dimensions. Secondly, if the polymer intermolecular attraction forces can be overcome through the formation of strong polymer–solvent interactions the gel will rapidly disintegrate to form a true solution.

As a result of strong intermolecular attraction forces and the low degree of dissociation of H^+-ion saturated humic substances, the higher molecular weight components can only swell in water. However, the Na^+, $NH_4{}^+$-polymer salts are dissociated in water and the resulting repulsive forces between the negatively charged groups on the polymer are generally far stronger than the intermolecular attractive forces. Such repelling effects cause flexible macromolecules to assume shapes, or conformations, which minimize the electrostatic free energy and lead to very large expansions of the polymer structures. Such expansions allow free access of solvent to the absorption sites and solution takes place.

As the pH of the extracting medium is raised increased ionization of the different acid functional groups (largely carboxylic acid and phenolic hydroxyls) gives greater repulsion and increased solubility. However, solution of high molecular weight polyelectrolytes (charged, or ionizable polymers) will only take place when sufficient ionized species are present to cause significant changes in polymer conformations. Also, highly cross-linked polymers, even if extensively charged, will not dissolve because they cannot assume expanded conformations.

3.2.2 Extractions with Base, Complexing Agents, and Neutral Salt Solutions

Achard (1786) was first to isolate humic acid when he extracted a peat soil with aqueous sodium hydroxide. This is still the most widely used solvent

system for humic substances. However, a certain amount of oxidation of humate, as measured by increases in the oxygen contents of the extracts (Bremner, 1950; Swift and Posner, 1972), takes place in the alkaline media. Nowadays, when sodium hydroxide solutions are used, the soil-solvent medium is flushed with nitrogen, extraction is carried out in an atmosphere of nitrogen, and sometimes reducing substances such as stannous chloride (Choudri and Stevenson, 1957) are added. Such laboratory practices can be expected to decrease the formation of artefacts, and are encouraged by the chemical, spectroscopic, and gel filtration data of Schnitzer and Skinner (1968). These indicate that the composition and properties of fulvic acids (see Section 3.3.1) are not significantly changed by solution contact with the base in an atmosphere of nitrogen. However, fulvic acids are the most highly oxidized of the polymeric humic substances.

Bremner and Lees (1949), in their search for solvents which would produce the least amounts of humic artefacts, showed that sodium and potassium salts of inorganic and of organic acids could be used to extract significant yields of humic substances. Of these the 0.1M solution of sodium pyrophosphate ($Na_4P_2O_7$), neutralized by addition of phosphoric acid, was best. This was followed, in decreasing order of efficiency for the sodium salts of the inorganic series, by the fluoride, hexametaphosphate $(NaPO_3)_6$, orthophosphate (Na_3PO_4), borate ($Na_2B_4O_7$), chloride, bromide and iodide. The order of decreasing extraction efficiencies of the organic salts was oxalate [$(CO_2Na)_2$], citrate [$(NaO_2CCH_2C(OH)(CO_2Na)CH_2CO_2Na$], tartrate [$NaO_2CCH(OH)CH(OH)CO_2Na$], malate [$NaO_2CCH(OH)CH_2CO_2Na$], salicilate (*o*-hydroxy-$C_6H_4CO_2Na$), benzoate ($C_6H_5CO_2Na$), succinate ($NaO_2CCH_2CH_2CO_2Na$), Na-4-hydroxybenzenecarboxylate, and ethanoate (CH_3CO_2Na).

The best extractants in the foregoing list form complexes with polyvalent metals bound to humic materials, and allow sodium ions to associate with the polyanions. Also the solubility is enhanced by the breakdown of the pseudo-cross-linking effects of polyvalent metals bridging charged functional groups on polymer chains. After removal of these metal ions the extents to which humic substances dissolve depends especially on the numbers and the extents of dissociation of their acid functional groups, and on the molecular weights of the polymers. However, solution will be influenced by the amount of low molecular weight electrolyte present. Additional electrolyte increases the ionic strength outside the polymer coil relative to that inside it, and decreases the thickness of the electrical double layer (see Chapter 5) causing the structure to contract. At high salt concentrations polymer concentration can be such that solution will not take place (see Section 3.5.5).

Alexandrova (1960) has shown that sodium pyrophosphate complexed calcium to such an extent that pretreatment of serosems (soils with high calcium contents) with acid prior to extraction was not necessary. Indeed, pretreatment could be expected to have a depressing effect on the solubility of humic substances in aqueous solutions of complexing agents because of the low degrees of

Table 3.1 Yields (%) and electron spin resonance (e.s.r.) data for humic (HA) and fulvic (FA) acids extracted by different solvents from a H^+-saturated organic soil (from Hayes *et al.*, 1975a). Reproduced by permission of Elsevier Scientific Publishing Co.

Solvent	% Yield			e.s.r. data (Spins $g^{-1} \times 10^{-16}$)		
	HA	FA	Total	HA	FA	H+FA
2.5M EDA	49.0	14.0	63.0	15.0	27.5	
EDA (anhydrous)	2.0	3.0	5.0	6.4	12.8	
0.5N NaOH	58.0	2.0	60.0	4.6	0.4	
0.1M $Na_4P_2O_7$	13.7	0.8	14.5	4.5	1.9	
1M Na^+-EDTA	12.5	3.8	16.3	0.3	0.3	
Pyridine	34.0	2.0	36.0	n.d.	n.d.	2.1
DMF	16.0	2.0	18.0	n.d.	n.d.	1.4
Sulpholane	10.0	12.0	22.0	n.d.	n.d.	1.0
DMSO	17.0	6.0	23.0	n.d.	n.d.	4.2

EDA = ethylenediamine; EDTA = ethylenediaminetetraacetic acid; DMF = dimethyl formamide; DMSO = dimethylsulphoxide; n.d. = not determined.

ionization of hydrogen-ion saturated (H^+-) humic substances. This is illustrated for extractions with pyrophosphate ($Na_4P_2O_7$, neutralized to pH 7.0 with phosphoric acid) and the sodium salts of ethylenediaminetetraacetic acid (EDTA) in Table 3.1. The poor solvent effects of these compounds for a H^+-organic soil can be explained by the fact that most of the polyelectrolytes were not significantly expanded in the unbuffered, and hence acidic media. The extractability in sodium hydroxide is generally significantly improved by acid pretreatment and removal of the excess acid, but Kononova and Bel'chikova (1961) have presented evidence which is widely accepted, to show that prior decalcification of soil with acid can be avoided by using an aqueous solution of 0.1M pyrophosphate and 0.1M NaOH.

3.2.3 Extraction with Organic Bases

Dryden (1952) has drawn attention to the usefulness of primary aliphatic amines, and particularly ethylenediamine (1,2-diaminoethane) or EDA, as solvents for brown- and low rank vitrain-type coals. He reasoned that the unshared electron pairs on the nitrogen atoms can partially accept a hydrogen atom attached to another molecule to form a hydrogen bond, and this would be the basis for a good solvent effect, as outlined among the criteria of Whitehead and Tinsley (1964).

Hayes *et al.* (1975a) have investigated the solubilities in anhydrous EDA, aqueous 2.5M EDA, sodium pyrophosphate, EDTA, pyridine and the dipolar aprotic solvents, dimethylformamide (DMF), sulpholane, and dimethyl-sulphoxide (DMSO). Aqueous EDA, with a medium pH of 12.6 (Table 3.2) extracted the greatest total quantity of humic materials, and this can be attributed

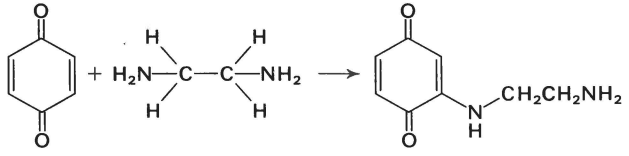
Table 3.2 Sequential extraction of H^+-organic soil samples using water and 1/1 solvent–water mixtures (after Hayes *et al.*, 1975a). Reproduced by permission of Elsevier Scientific Publishing Co.

Solvent[a]	Yield[b] (% of organic matter)			pH values	
	HA	FA	Cumulative	Extractant	Extract
Water	0.0	2.8	2.8		
DMF	15.0	2.2	20.0	6.8	2.9
Sulpholane	4.1	1.0	25.1	3.7	1.2
DMSO	0.7	0.2	26.0	5.9	1.3
Pyridine	14.8	0.6	41.0	11.6	4.2
EDA	23.2	6.3	70.9	13.0	12.6

[a] Solvent symbols are the same as those used in Table 3.1.
[b] HA = humic acids; FA = fulvic acids.

to the basicity of the medium, and to the ability of the organic compound to link to the polymer. However, most of the fulvic acid (FA) materials precipitated during dialysis. This precipitate was soluble in sodium hydroxide and reprecipitated on acidification to pH 1.0, and it would normally be classed as humic acid (see Section 3.3.1).

Table 3.3 provides evidence that EDA significantly increases the nitrogen contents of humic substances, and this could not be lowered further by repeated washings with, or dialysis against dilute acid solutions. Dryden (1952), Rybicka (1959) and others have observed the same behaviour for coal extracts, and the nitrogen is thought to be incorporated by formation of covalent linkages between EDA and quinone-type structures in the humic polymers, as illustrated in reaction scheme (3.1).



(3.1)

Anhydrous EDA is a poor solvent for dried organic matter (Table 3.1). This emphasizes the importance of hydrating the anionic species in the polyelectrolyte. Also this organic molecule would have difficulty in penetrating into the dry polymer matrix held together by van der Waals and hydrogen bonding forces.

Pyridine, with a pK_b value of 8.75 is a very much weaker base than EDA (pK_{b1} = 3.3, pK_{b2} = 6.44) and accordingly it is a poorer solvent than EDA for humic substances (note the pH of H^+-humic substances in pyridine, Table 3.2). Again, like EDA, it incorporated nitrogen into the polymers (Table 3.3), but it is very likely that this resulted from adsorption and not from the formation of covalent linkages. Adsorption of heterocyclic aromatic nitrogen compounds by humic substances is well known in the pesticide field (Hayes, 1970).

Table 3.3 Elemental composition (%) of humic acids (HA) and fulvic acids (FA) extracted with different solvents from an H^+-organic soil (after Hayes *et al.*, 1975a). Reproduced by permission of Elsevier Scientific Publishing Co.

Solvent[a]	C		O[b]		H		N		S	
	HA	FA	HA	FA	HA	FA	HA	FA	HA	FA
2.5M EDA	56.8	51.2	29.2	30.3	5.9	5.7	6.4	11.1	n.d.	n.d.
Pyridine	55.9	47.1	32.9	39.9	5.1	5.3	4.4	6.0	n.d.	n.d.
DMSO	55.0	55.0	35.5	37.1	4.2	4.4	3.3	2.2	2.0	1.3
Sulpholane	54.4	53.2	35.2	37.4	4.8	4.4	3.2	3.3	2.4	1.7
DMF	54.3	52.3	36.8	38.8	4.6	4.1	2.6	3.2	1.7	1.6
0.5N NaOH	53.1	45.0	36.3	43.0	6.0	6.0	2.9	4.3	n.d.	n.d.
Na^+ EDTA	52.1	48.4	—	—	4.1	4.2	n.d.	n.d.	n.d.	n.d.
0.1M $Na_4P_2O_7$	50.9	37.3	41.1	50.9	3.3	5.1	3.0	5.0	n.d.	n.d.

[a] Solvent symbols are given in Table 3.1.
[b] Where not determined, *S* values are assumed to be 1% for estimation of % O by difference; n.d. = not determined.

3.2.4 Extraction with Dipolar Aprotic Solvents

Because the acid groups of H^+-soil organic materials are largely undissociated, humic substances will have solubility properties in dipolar aprotic solvents (see pH values of extracts, Table 3.2) similar to those of a number of neutral polymers. For solution to take place a decrease must take place in the Gibbs free energy (G) of the system, and the free energy of mixing, expressed by the relationship

$$\Delta G_m = \Delta H_m - T\Delta S_m$$

will be negative (where T is the temperature, K; $\Delta H_m = -\Delta Q$ refers to the heat of mixing; ΔS_m is the change in entropy and is related to the increase in freedom of movement or disorder in the solution). The heat of mixing may be represented by the semiempirical Scatchard–Hildebrand equation (as outlined by Hildebrand and Scott, 1962)

$$\Delta H_m = V(\delta_1 - \delta_2)^2 \phi_1 \phi_2$$

where V is the total molar volume of solution, and δ_1 and δ_2, and ϕ_1 and ϕ_2 are solubility parameters and volume fractions, respectively, of solute and of solvent.

A negative value for ΔG_m is assured if the enthalpy value ΔH_m is small or negligible, and this will happen when the intermolecular interactions between solvent–solvent and polymer–polymer molecules are similar. This similarity in intermolecular interactions reflects similarities in δ values, and is an indication of what is known as the *cohesive energy density* (CED) value of the system. The CED measures the energy required to create a 'hole' in the solvent large enough to accommodate the solute and to promote solvation.

Enthalpies of mixing will approach zero when the solubility parameters are similar. Seymour (1971) has pointed out that, provided solvents are not dis-

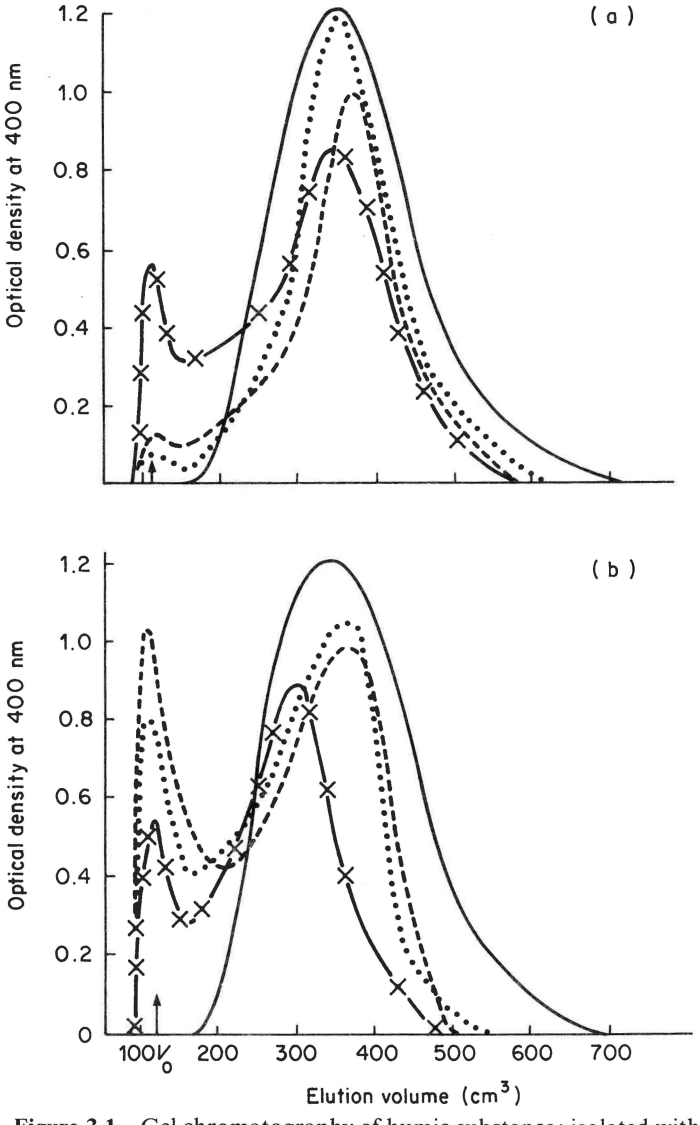


Figure 3.1 Gel chromatography of humic substances isolated with dimethyl formamide (———), pyridine (- - - - -), dimethylsulphoxide (· · · · · ·), and ethylenediamine (×———×———×) in single (a) and sequential (b) solvent extraction. (After Hayes *et al.*, 1975a.) Reproduced by permission of Elsevier Scientific Publishing Co.

similar in molecular structures, the solubility parameters of a solvent mixture will generally be equal to the sum of the products of the mole fractions and solubility parameters of each component. For that reason polymers which are insoluble in individual solvents may be soluble in mixtures of these.

The data in Table 3.1 shows that the total amounts of organic matter extracted

by the three dipolar aprotic solvents were similar although the quantities of fulvic acids extracted were different. In addition the elemental compositions of the different humic and fulvic acids were very similar (Table 3.3) and these data indicate that the fulvic acids were less highly oxidized than those obtained by sodium hydroxide and pyrophosphate solutions. The solubility parameters (δ values) for DMF and DMSO (Tobolsky and Yannas, 1971) are nearly the same (12.4 and 12.93, respectively), which might partially explain the comparable solubilizing effects of the two solvents.

Figure 3.1a shows that, compared with EDA, only a trace of DMSO and none of the DMF extracted humic acids were of sufficiently high molecular weight to elute at V_0 (the void volume) during gel chromatography on Biogel P300 (see Section 3.5.2). Therefore it would appear that the dipolar aprotic solvents preferentially extracted the less highly oxidized and lower molecular weight fractions of humic substances. Such solvents may therefore be of value for selectively extracting relatively homogeneous humic materials.

3.2.5 Sequential Extraction Procedures

Table 3.2 provides data for yields of humic materials sequentially extracted with water, dipolar aprotic solvents, pyridine, and EDA. The technique involved exhaustively extracting with a single solvent system before proceeding to the next in the series. Similarities in the solution properties of the dipolar aprotic solvents is emphasized by the low yields extracted by DMSO in particular, which was preceded in the solvent series by DMF and sulpholane. Figure 3.1b shows that, as expected, the molecular weight distribution of extracted humic acid in the first organic solvent used was the same as for the single extraction (Figure 3.1a.) However, the amounts of high molecular weight materials from the subsequent extractions with DMSO and pyridine were increased. It is clear too that most of the lower molecular weight materials extractable in EDA were removed by the earlier solvents used. These data suggest that sequential extraction presents a further refinement of procedures for obtaining less heterogeneous fractions of humic materials.

3.2.6 Artefact Formation

Reference has been made to oxidations of humic substances during extractions with sodium hydroxide (particularly under aerobic conditions) and to the additions of nitrogen from EDA and pyridine solvent systems. Electron spin resonance (e.s.r) data (Table 3.1) suggest that free radicals were formed in the polymers (see Section 3.5.1) during contact with EDA. There is, however, no evidence for artefact formation during extractions with dipolar aprotic solvents and EDTA, and none would be expected during solution in pyrophosphate.

Oxidation of phenols to quinones can proceed by a free radical intermediate mechanism, but comparisons between e.s.r. data for the pyrophosphate and

sodium hydroxide extract (Table 3.1) suggest that stable free radicals were not formed during contact with the base. Therefore it would appear that sequential extractions with non-polar solvents to remove waxes, followed by dipolar aprotic solvents, pyrophosphate in neutral media, and 0.5N sodium hydroxide would provide a good solvent series for the isolation of humic substances.

3.3 FRACTIONATION AND PURIFICATION OF HUMIC EXTRACTS

Brief reference has already been made to the heterogeneity of composition of soil organic matter extracts. Thus it is common practice to follow extraction by fractionation procedures in order to obtain materials which are less heterogeneous than the original extract and which can be characterized to some degree.

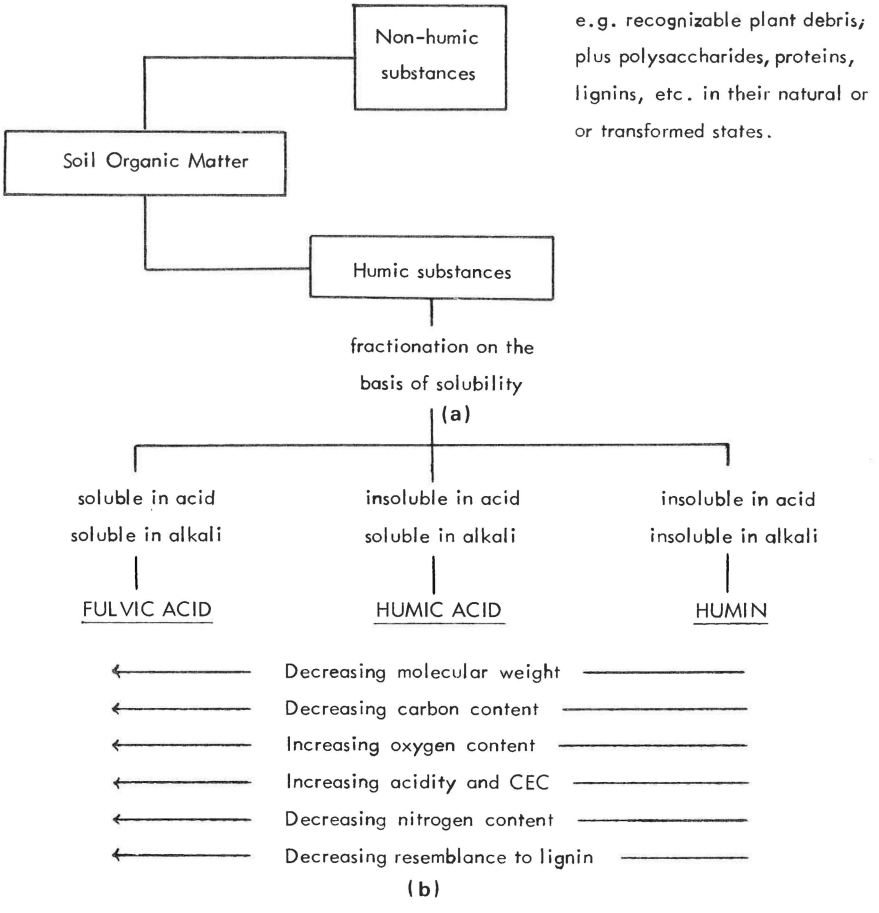


Figure 3.2 Fractionation of soil organic matter and humic substances (a), showing (b) how some properties vary in the fulvic acid–humin range

Such fractionations sometimes fulfil a dual purpose because the same techniques often allow the removal of some non-humic substances and hence permit a degree of purification to be achieved. In general the techniques used for purification and fractionation attempt to exploit differences in solubilities, adsorption behaviour, and molecular weight or charge characteristics either between the humic and non-humic substances, or between the different components of the humic substances.

3.3.1 Fractionation Based on Solubility Differences

Classically, the fractionation of the humic substances is based on differences in solubilities in acid and in alkaline media. The techniques involve extraction with an alkaline reagent, separation of the soluble extract from the soil residue, and then acidification of the extract. This procedure gives rise to three gross fractions (Figure 3.2a) called *fulvic acids*, soluble in acid and in alkali; *humic acids*, soluble in alkali but precipitated by acid; and *humins*, insoluble in acid and in alkali. Some workers further subdivide humic acids by addition of salt to give *gray* and *brown humic acids*, or by extraction of alcohol soluble *hymatomelanic acids*. The same nomenclature is used for the three main fractions even when the extraction is carried out using neutral salts, complexing agents, or organic solvents in place of the traditional alkali.

The classical fractionation procedures are discussed in detail by Kononova (1966) and by Stevenson and Butler (1965). Because of the nature of the extraction and fractionation processes it is inevitable that certain non-humic impurities will accumulate in particular fractions. Thus, for example, some polysaccharides and numerous low molecular weight compounds are found in the fulvic acid fraction, highly lignified materials are found in the humic acid fraction, and inorganic contaminants, including colloidal materials and some salts are common to all fractions.

3.3.2 Purification of Humic Fractions

Humic extracts from mineral soils are generally contaminated with silicates and salts, and further quantities of the latter are generated during acidification of inorganic reagent extracts. Silicates can be dissolved by treatment with HF or with HCl–HF mixtures. Mortensen and Schwendinger (1963) effectively removed clay from their soil extracts in pyrophosphate by treating the fractions for 10 minutes at 4 °C with a solution 0.3 molar with respect to HCl and HF. The solution was then neutralized and dialysed. Dubach and Mehta (1963) have reviewed how preparations with very low ash contents can be obtained by repeated solution-centrifugation-precipitation techniques, or by treatment with complexing agents and ion-exchange resins, etc. Most of the non-polymeric organic materials, as well as salts, are removed during dialysis.

The fulvic acid fraction is composed of a variety of heterogeneous components, including polysaccharides (Section 3.6) which can be separated by adsorption on charcoal (Forsyth, 1947), or by use of gel and resin chromatography techniques (Barker *et al.*, 1967; Swincer *et al.*, 1968). In general humic acids are less contaminated by non-humic organic polymers and lend themselves more readily to purification procedures.

In many recent researches saccharide and protein or peptides are removed from humic acid by prolonged hydrolysis in 6M HCl. Decarboxylation takes place during hydrolysis (Deuel and Dubach, 1958a, 1958b), although Riffaldi and Schnitzer (1973) have indicated that charge, free radical and other properties of humic acids are not significantly affected by this treatment.

The three gross fractions of humic substances should not be regarded as distinct or discrete components, but should be considered as a spectrum of substances, or related molecules which display continuous variations with respect to certain properties. Amongst the properties which vary are molecular weight, cation–exchange capacity, elemental composition, etc. (Figure 3.2b). Each of these fractions can be refractionated to bring about further reductions in heterogeneity. The techniques generally employed are:

(i) electrophoresis, electrofocusing, and ion exchange—all of which exploit differences in charge and charge density;
(ii) gel chromatography which fractionates on the basis of molecular weight and size differences;
(iii) adsorption chromatography on a variety of media;
(iv) the further use of solubility differences, employing fractional precipitation with solvents, salting out and changes in medium pH.

Several of these procedures and their specific application to humic substances will be discussed in more detail in Section 3.5. Suffice it to say here that each of the techniques always produces a fractionation, but the different fractions can themselves be refractionated. Thus it will be difficult to isolate a single, homogeneous humic entity because such species are probably present in very low concentrations among related molecules in the polydisperse system. However, diligent application of fractionation techniques to humic substances enables the isolation of preparations with greatly reduced heterogeneity. This allows such preparations to be examined by a variety of physico-chemical techniques, but more important it permits more meaningful results to be obtained from structure (primary) and shape (tertiary structure) studies.

3.4 CHEMICAL COMPOSITION OF HUMIC SUBSTANCES

We define as chemical composition the elemental contents, and the numbers and types of functional groups (carboxyl, hydroxyl, etc.) and of organic molemolecules or 'building blocks' which make up the humic polymer structures. Elemental analysis data can be readily obtained by standard procedures, and

techniques have been developed which allow determinations to be made of many of the functional groups present. Difficulties are being experienced in finding suitable degradative reagents and conditions which allow the isolation of structurally meaningful organic molecules, although considerable progress has been made in this area of late.

Synthesis by chemical methods, and by microbial and enzymatic procedures offers alternatives to structural determinations by degradation reactions. Interest in non-enzymatic chemical synthesis has declined in recent times, although considerable research effort is now being directed to microbial and enzymatic procedures. These enable the disappearance of monomer units into the polymer structures to be followed, and the reappearance of the same units in digests after chemical (or enzymatic) degradation of the polymer is regarded as good evidence for their involvement in the 'humic-type' structures.

3.4.1 Elemental Analysis

Elemental or ultimate analysis involves the determination of the elements present in a compound. Dividing the percentage of each element in the compound by its atomic weight gives the ratios for the different elements present, and the compound's empirical formula can then be calculated by dividing all of the ratio values by the smallest of these. A knowledge of the molecular weight of the compound is required in order to determine its molecular formula, which will be some multiple of the empirical formula.

Elemental analysis and the data which it gives are used to check the purity of compounds and, together with chemical and spectroscopic data, to assign structures to organic compounds. In cases of complex structures, such as those of humic materials, ultimate analysis can only be interpreted in a very general way in structural terms, even when molecular weight values are available. They do, however, provide accurate values for the proportions of the elements in the different samples, and of course they allow comparisons to be made between samples.

Routine procedures are available for determinations of the carbon, hydrogen, oxygen, nitrogen, sulphur and ash contents; data for the elements are most useful when calculated on a dry, ash-free basis. Carbon and hydrogen are determined by combusting a weighed sample of material (usually in the presence of a CuO catalyst) in a stream of oxygen. The water and carbon dioxide given off are collected in anhydrous magnesium perchlorate [$Mg(ClO_4)_2$] and soda lime, respectively, and determined gravimetrically. Nitrogen is estimated from micro-Kjeldahl-, which uses selenium–mercury catalysts, or Dumas-digestion procedures, and the oxygen-flask method gives good determinations of the organic sulphur contents (MacDonald, 1965). Oxygen is determined by difference,

$$\%\,O = 100 - (\%\,C + \%\,H + \%\,N + \%\,S),$$

or by direct methods. The best of these involve conversion of the organically bound oxygen to CO and its determination by instrumental procedures (Schöniger, 1969), especially by gas–liquid chromatography (Boos, 1964). The accuracy of such methods is impaired when various metals and phosphate and fluoride ions are present; consequently they should only be applied to H^+-saturated humates which have low ash contents. Ash can be determined gravimetrically by combusting the substrate in a stream of oxygen in a furnace at 400–500 °C.

The data in Table 3.3 show how the elemental compositions of humic and fulvic acids are influenced by the solvent used to extract the organic matter source. Reference has already been made to the different solvating effects of the extractants used and to the close similarity between the compositions of the humic and fulvic acid materials extracted by the dipolar aprotic solvents DMSO, sulpholane, and DMF. On the other hand, the elemental composition data suggest significant differences between the humic and fulvic acid materials extracted under basic conditions or in the presence of the chelating agents.

Thus close similarity should not be expected between humic materials extracted with different solvents from the same or from a variety of soils. Where the same extractant is used, however, elemental composition data allow interesting comparisons to be made between humic substances from different soils and sources. Table 3.4 provides such data for substances extracted in alkali from soils and coals. The fungal humic acids listed were obtained as precipitates at pH 2.0 from filtered culture media which had been incubated for seven weeks with the *Stachybotrys* fungal species, *S. chartarum* and *S. atra*.

The sod podzolic humates were from northern USSR, the chernozems were from the Ukraine, the inceptisols from Dominica in the West Indies, and Table

Table 3.4 Elemental composition (%) of humic acids (HA) and some fulvic acids (FA) extracted by alkali from different soils and materials

Sample	% C		% O		% H		% N	
	HA	FA	HA	FA	HA	FA	HA	FA
Sod podzolic[a]	57.6	42.6	35.3	44.6	5.2	5.0	4.8	4.1
Chernozem[a]	62.1	44.8	31.4	49.4	2.9	3.4	3.6	2.3
Inceptisol[b] (surface)	54.5	42.8	34.1	47.7	5.3	3.8	5.5	2.0
Inceptisol[b] (sub surface)	51.4	46.9	36.0	44.6	6.7	4.5	4.9	2.3
Brown coal[c]	63.1		(32.7)		2.8		1.4	
Brown coal[d]	65.4				4.0			
S. chartarum[e]	57.3		27.7		7.0		6.8	
S. atra[e]	53.1		34.8		5.7		5.4	

[a] From Kononova (1961).
[b] From Griffith and Schnitzer (1976).
[c] From Lawson and Purdie (1966).
[d] From Yokakawa *et al.* (1962).
[e] From Schnitzer and Neyroud (1975).

3.4 shows a carbon content maximum for the humates from the intermediate latitude. There was a close similarity in the composition of the chernozem humic acid and that of the brown coal from central England described by Lawson and Purdie (1966). Clearly there are only about half as many hydrogen as carbon atoms in these structures, and this could indicate extensive substitution or branching. The oxygen contents listed for all of the soil fulvic acids indicate that these compounds were highly oxidized.

Elemental analysis for the fungal 'humic' acids are presented for purposes of comparison. Inspection of the data for the *S. atra* product shows that its elemental composition is of the order of that for the inceptisol surface and subsurface soil humic acids. Those for *S. chartarum* are less comparable with the usual values for the soil humic substances.

Conclusions should not be reached about similarities between humic substances on the basis of ultimate analysis data alone. It would for instance, be highly erroneous to infer from the data in Table 3.4 that the humic acid material from *S. atra* is similar to that from either of the inceptisol samples, or that those from the chernozem and brown coal are the same. Such statements could only be made with any degree of conviction if the same functional groups were present in more or less the same abundances, if the numbers and types of degradation products from specific degradation reactions were the same, if the molecular weight distributions were closely similar, and if the shapes of the component molecules were comparable. It is very unlikely that all of these requirements for comparability would be met by any two highly polydisperse humic products from different soil or source types. For valid comparisons to be made it would be necessary to analyse products with decreased polydispersities by additional procedures which will be described in Section 3.5.2.

3.4.2 Determinations of Functional Groups

The numbers and the types of oxygen-containing functional groups greatly influence the reactivities of humic substances in the soil environment. Undoubtedly the carboxyl and phenolic structures are the most important of these because they are the major groups responsible for the contribution by organic matter to the cation-exchange capacity (CEC) of soils, and they can have chelating effects (Schnitzer and Skinner, 1965a) which might influence plant nutrition. Most of the carboxyl groups dissociate to the carboxylate anion at the pH values likely to be encountered in fertile soils. Phenol and hydroxybenzene derivatives containing activating substituents on the aromatic nucleus are not substantially dissociated when the pH is less than 9.0, but deactivating substituents can sufficiently lower the pK_a values to allow them to contribute to the soil CEC. Hydroxyquinone structures are also highly acidic although it is not certain to what extent, if any, they are present in humic substances.

It is not surprising, therefore, that substantial interest and research effort has been focused on investigations of the types and numbers of oxygen-containing

functional groups in humic substances. It will be seen that accurate measurements and interpretations of data are sometimes difficult because of the complex chemical and steric environments of these groups.

Oxygen-containing functional groups

Carboxyl, phenolic hydroxyl, alcoholic hydroxyl, carbonyl and possibly quinone, and methoxyl groups are considered to be the principal oxygen-containing functional groups in humic substances. It is highly probable that additional ether groups, and in the light of recent researches some ester groups are also present.

Mention has been made of the complex chemical environments of the functional groups, and of the contributions of the carboxyl and of phenolic hydroxyl groups to the acidities of humic materials. Enolic structures can also be acidic and this adds a further complication to the analytical problem because none of these groups can be determined accurately by pK dependent techniques such as titration. This can be explained on the basis of the ranges of pK_a values for each group which result from the influences of neighbouring groups and from steric effects.

Determination of total acidity. Two methods have become generally accepted for this determination. The first involves the use of diborane (B_2H_6), which liberates gaseous hydrogen from even sterically hindered acidic groups in H^+-ion saturated preparations, and was used by Martin *et al.* (1963) to determine the total acidity in fulvic acid from a podzol B_h horizon. Their results were in good agreement with the second, or the barium hydroxide method.

The $Ba(OH)_2$ method was developed by Brooks and Sternhell (1957) to determine the acidity in preparations of brown coal, and it was first applied to soil humic materials by Wright and Schnitzer (1959a, 1960). When reacted with H^+-saturated humic substances the following reaction takes place:

$$Ba(OH)_2 + 2H^+\text{-humate} \longrightarrow Ba^{2+}(\text{humate})_2 + H_2O$$

A modification of the procedure by Brooks and Sternhell was described by Schnitzer and Gupta (1965) and is also presented in Schnitzer and Khan (1972). In practice an excess of $Ba(OH)_2$ is reacted with the humic materials and the unreacted base is back-titrated with standard acid. It is assumed that all of the exchangeable hydrogen ions are replaced by Ba^{2+}. This assumption need not always be valid, however. For instance Schnitzer and Gupta (1965) found that some substituted phenols did not quantitatively replace acidic hydrogen with barium ions, yet the $Ba(OH)_2$ procedure gave values for the total acidity of humic substances which were in close agreement with those for the diborane method. Similar low replacement effects might be expected for the humic substances because of steric effects which could prevent access to all of the

exchange sites. On the other hand, barium taken up by chelation or complex formation would lead to falsely high values for total acidity.

It is, of course, possible to determine total acidity directly by titration of H^+-humic materials with strong base. The spread of pK_a values for the acidic groups of the polymers complicates this procedure, however, because it is difficult to locate the end point on the titration curve. Arbitrary end-points are taken at pH values of 7.0 or 7.5, and the acidities calculated from such titration data are reasonable indications of the sum of the contributions which the various acidic groups can make to the CEC of the soil. It is necessary to carry out titrations to higher pH values in order to estimate the total acitity of the sample being investigated.

Diazomethane reacts with functional groups containing acidic hydrogens to give ester products from carboxylic acids and methyl ethers from phenolic hydroxyls, as shown in reaction scheme (3.2)

$$\begin{aligned} RCO_2H + CH_2N_2 &\longrightarrow RCO_2CH_3 + N_2 \\ PhOH + CH_2N_2 &\longrightarrow PhOCH_3 + N_2 \end{aligned} \qquad (3.2)$$

where Ph represents a phenyl group. The total acidity can then be estimated from the increase in the $-OCH_3$ content. Schnitzer (1974a) found that the acidity of seven humic acid samples measured by this procedure ranged from *ca.* 65–97% of that determined by $Ba(OH)_2$.

Determination of carboxyl groups. Carboxyl groups are the major contributors to the acidic properties of humic substances in what might be termed soils of normal pH values. The presence of the carboxyl group has been demonstrated by several workers who observed shifts in the maxima of the carbonyl bands from 1700–1750 cm^{-1} for the undissociated carboxyl to 1600–1650 cm^{-1} for dissociated carboxyl groups (e.g. Farmer and Morrison, 1960; Martin *et al.*, 1963; Theng and Posner, 1967), and by the disappearance of the carboxyl band after treatment with diborane (Martin *et al.*, 1963).

The amounts of carboxyl present in humic substances are generally determined by the calcium acetate method, described by Schnitzer and Gupta (1965), in which the carboxyl of H^+-humate reacts with excess calcium acetate, as shown in reaction scheme (3.3).

$$2RCO_2H + (CH_3CO_2)_2Ca \longrightarrow (RCO_2)_2Ca + 2CH_3CO_2H \qquad (3.3)$$

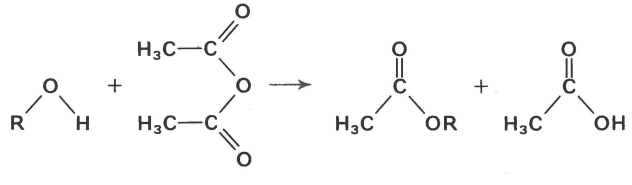
The released ethanoic acid is measured by titration. To be effective the technique requires that Ca^{2+} ions should have access to and be able to exchange with all of the hydrogens on the carboxyl groups, and that exchange should not take place with hydrogen ions from the acidic phenolic groups. Because calcium acetate reacts with the more readily dissociated phenolic groups, as shown in experiments with model substances by Schnitzer and Gupta (1965), the method might be expected to give overestimates of carboxyl numbers. However, the

bridging by calcium between carboxylate groups on different molecules (or randomly between such groups on the same polymer molecule) gives rise to aggregated and more compact structures and increases the difficulties of diffusion to and from the exchange site (see Sections 3.5.4 and 3.5.5). Such structural modifications would inhibit reaction with carboxyl groups in the interior of the aggregates, but these effects might be compensated by exchange with appropriate available phenolic or enolic structures to give, fortuitously, carboxyl values comparable with those obtained by other methods.

An alternative procedure uses $CuSO_4$–quinoline reagent (Hubacher, 1949) to decarboxylate the carboxyl groups. In theory this should be a more accurate method. Schnitzer and Gupta (1965) found that results from this method agreed well with those from the calcium acetate procedure.

Determination of phenolic hydroxyls. Despite inherent inaccuracies, the amounts of phenolic hydroxyl in humic substances is generally assessed as the difference between the total acidity and the carboxyl group contents. Wright and Schnitzer (1960) have described a modification of the procedure of Ubaldini and Siniramed (1933) which involves refluxing the humic material with alcoholic KOH. After removal of the excess base the residue is equilibrated with an 85 % ethanol solution saturated with CO_2. Potassium released by the ethanol treatment, determined as K_2CO_3 by titration, is considered to be displaced from phenolic groups only. However, the technique cannot be relied upon for use with humic substances which have phenolic structures with a spread of pK_a values, and it is thus no more attractive than the difference method.

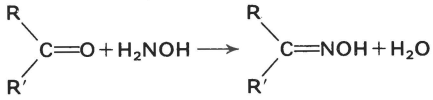
Determination of total hydroxyls. Total hydroxyl group content can be estimated following acetylation by means of acetic anhydride in pyridine (Brooks *et al.*, 1957), as shown in reaction scheme (3.4). Then the ester product formed can be separated, hydrolysed in base, and the ethanoic acid steam distilled from the acidified mixture and estimated by titration. Alternatively, a less favoured procedure involves determination of the ethanoic acid released during aqueous titration of the excess acetic anhydride (Wright and Schnitzer, 1960).



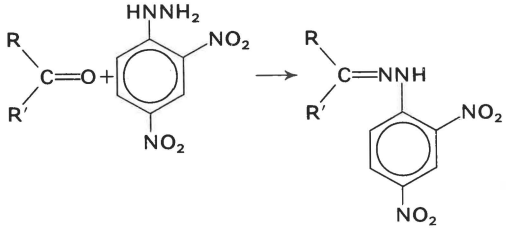
(3.4)

Determination of alcoholic hydroxyls. As yet there is no satisfactory procedure for the determination of alcoholic hydroxyl groups and these are usually unreliably estimated as the difference between the total hydroxyl and the phenolic hydroxyl contents.

Determination of carbonyl and quinone groups. Values for the carbonyl contents of humic substances can be obtained by determining the increases in nitrogen contents of humate derivatives formed by reactions with hydroxylamine (Flaig, Scheffer and Klamroth, 1955; Schnitzer and Skinner, 1965), phenylhydrazine (Schnitzer and Skinner, 1965), or 2,4-dinitrophenylhydrazine (Flaig *et al.*, 1955; Schnitzer and Skinner, 1965). The generalized reactions for hydroxylamine and 2,4-dinitrophenylhydrazine are outlined in reaction scheme (3.5) where R represents aliphatic or aromatic humic substituents and R′ would ideally be similar type structures, or hydrogen.



(3.5)

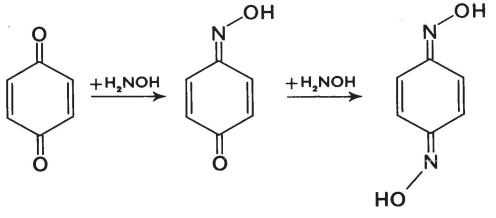


Other procedures determine the amounts of unreacted reagents (Brooks *et al.*, 1958; Schnitzer and Skinner, 1966). In this context Schnitzer and Skinner (1966) developed a polarographic procedure for the determination of carbonyl groups in humic substances. The 2,4-dinitrophenylhydrazone derivative was prepared, as outlined in scheme (3.5), by refluxing the substance in an excess of 2,4-dinitrophenylhydrazine reagent, and the carbonyl content was estimated from the amount of unreacted reagent, which could be measured from the polarographic reduction of the nitro groups.

Flaig and his colleagues (see Flaig, Beutelspacher and Rietz, 1975, pp. 75–95) have placed considerable emphasis on considerations of polyphenols and of polyquinones in humic structures. Quinones can form charge-transfer complexes, and this might provide a mechanism for the binding of some pesticides by humic substances (Hamaker and Thompson, 1972). They could also be involved in forming metal humate complexes and in hydrogen bonding. Foster and Foreman (1974) have reviewed the chemistry and the detection of various complexes of quinone structures.

It is unfortunate, in view of their probable presence and importance, that satisfactory procedures are not available for the unambiguous identification and the quantitative determinations of quinone groups in humic structures. A study of a review by Finley (1974) will show that reactions based on the formation of Schiff base structures with aldehyde and keto groups will also

take place with quinone structures. Thus either the mono- or the dioxime can be formed from 1,4-benzoquinone as seen in scheme (3.6). However, the amount of oxime obtained will be governed by the extent of substitution of the quinone structure, and tetrasubstituted 1,4-benzoquinones will not form oximes for steric reasons. In addition substitution reactions, as shown in scheme (3.1), to give non-hydrolysable products can take place.

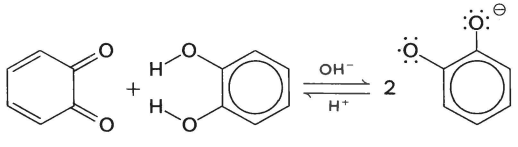


(3.6)

Obviously, from considerations of the foregoing reactions, it is not possible to differentiate between aldehyde-, keto- and quinone-carbonyl functional groups by estimating the increases in humic nitrogen contents from the formation of oximes or of derivatives of substituted hydrazines.

Berger and Rieker (1974) have stressed the usefulness of spectroscopic methods for the identification and quantitative determinations of quinones, and they have outlined in considerable detail the influences of adjacent carbon substituents on the quinone carbonyl vibration frequency in the 1630–1700 cm^{-1} region of the infrared spectrum. So far, however, spectroscopic techniques have failed to resolve the presence or otherwise of quinone structures in soil humic substances, although some success has been achieved with coal and coal humic acids (Brown and Wyss, 1955; Moschopedis, 1962; Fujii, 1963; Bailey *et al.*, 1965).

The best spectroscopic evidence for the presence of quinone structures in soil humic acids has been provided by electron spin resonance (e.s.r.) techniques (Section 3.5.1). Atherton *et al.* (1967) obtained spectra for a variety of acid-boiled humic acids and all had band breadths of the order of 1.75–1.9 gauss. The signals were eliminated when the samples were reduced with sodium dithionite but recovered when re-exposed to air. This could indicate the presence of semiquinone ion radicals, as was suggested by Steelink *et al.* (1963) and reviewed by Steelink and Tollin (1967). They suggest that polymers containing *ortho*- and *para*-quinhydrones would, under basic conditions give rise to the radical ions as indicated for *ortho*-quinhydrones in reaction scheme (3.7).

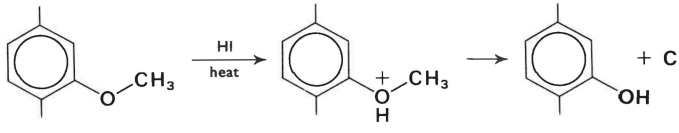


(3.7)

The reviews of Berger and Rieker (1974) and Finley (1974) draw attention to the usefulness of polarography for qualitative and quantitative studies on quinones. Flaig *et al.* (1969) have found linear correlations between the intensity of the infrared absorption of quinone carbonyls and the polarographic pulse heights at their half-wave reduction potentials. Unfortunately, as was pointed out earlier, only weak infrared absorptions indicative of soil humic acid quinones have been reported, and evidence for quinones from the polarography of humic acids is even weaker still. Should a variety of quinone structures be present, as seems likely, it is highly probable that these would have a range of half-wave reduction potentials and single, or well-separated polarographic reduction peaks would not be observed. Clearly considerable further experimentation, involving a range of buffers and a variety of treatments and of reaction conditions will be required before polarography can be properly evaluated for estimations of quinone structures in soil humic substances.

According to Vasilyevskaya *et al.* (1971) quinone structures can be quantitatively estimated from data obtained when humic substances are dissolved under N_2 in 0.1M NaOH, and solutions (2.5M) of NaOH and $SnCl_2$ are added until the final NaOH concentration is *ca.* 1.3M. If the reaction is allowed to take place for one hour at room temperature reduction of the quinone structures takes place and the unused $SnCl_2$ can be back-titrated with standard $K_2Cr_2O_7$ solution. Titanometric reduction (van Krevelen, 1961, pp. 160–176) has been used to determine quinones in coals and coal humic acids. This involves reduction, in a nitrogen atmosphere, with titanous chloride in acetic acid–hydrochloric acid (5:1), and oxidimetric titration of excess titanous chloride with ferric chloride.

Determination of methoxyl groups. Modifications of the Zeisel hydriodic acid procedure are generally used to determine methoxyl groups in lignins and in humic substances. The reaction involves boiling with HI. Iodomethane is liberated as indicated for an aromatic methyl ether structure in reaction scheme (3.8). Iodine, released from the iodic acid generated by oxidation of iodomethane, can be determined titrimetrically. Methoxyl content is highest in freshly humified plant tissue and it decreases as the humic substances become older.



(3.8)

Distribution of oxygen among the functional groups in humic substances. Only in the case of fulvic acids does the sum of the oxygen in the carboxyl, phenolic and other hydroxyl, carbonyl, and methoxyl groups approach 100% of the

oxygen contents of the humic fractions. Similar sums for brown coal- and soil humic acids, and humins range from 60–90 % of the total. Most of the oxygen of fulvic acids (the most highly oxidized component of humic materials) is in the carboxyl groups. This is generally true for humic acids also, but phenolic hydroxyls form an increased proportion of the total oxygen in these structures. Differences in the amounts of oxygen which can be assigned to detectable functional groups provide good evidence for the fact that humic structures formed under different environments can vary greatly. The unassigned oxygen might be bound, for instance, in heterocyclic structures or as phenolic ether linkages which would not be detected by the analytical procedures described. An ability to ascribe all of the oxygen to functional groups or organic structures would have an important bearing on predictions of humic polymer structures.

Nitrogen-containing functional groups

There are numerous reviews which deal with various aspects of the distribution of nitrogen in soil environments. Those by Bremner (1967) and Parsons and Tinsley (1975) are especially relevant to the contexts of this chapter.

Between 20 and 50 % of soil nitrogen is held as amino acids and 5–10 % as amino sugars (Bremner, 1967). Although amino sugars can be components of soil polysaccharides (Section 3.6) the amino acids cannot be accounted for as components of protein structures, but it is highly probable that peptide groups are present.

Hydrolytic procedures release amino acids and amino sugars, and these can be separated by use of buffers and ion-exchange resin techniques. There is an abundance of quantitative data, obtained by standard amino acid analyser techniques, for the amino acid composition of humic materials. One plausible theory proposes that much of the non-hydrolysable nitrogen is covalently linked to aromatic structures, introduced by electrophilic substitution of quinone structures as indicated by reaction scheme (3.1). Peptides could be linked in this way through the terminal amino group, or through the free-NH_2 group of diamino amino acids.

There is some evidence also for pyrrole-type structures and for some heterocyclic nitrogen in fused aromatic structures, e.g. acridine, which has been detected by zinc-dust distillation and fusion degradation processes (Section 3.4.3).

3.4.3 Degradation of Humic Substances

In order to determine the primary structure or the 'building blocks' of any naturally occurring polymer it must be degraded to the monomer units of which it is composed. This is relatively simple where the monomers are linked by labile bonds, such as the peptide bond in proteins, or glycosidic linkages in polysaccharides, since these are susceptible to hydrolysis in acid or alkaline

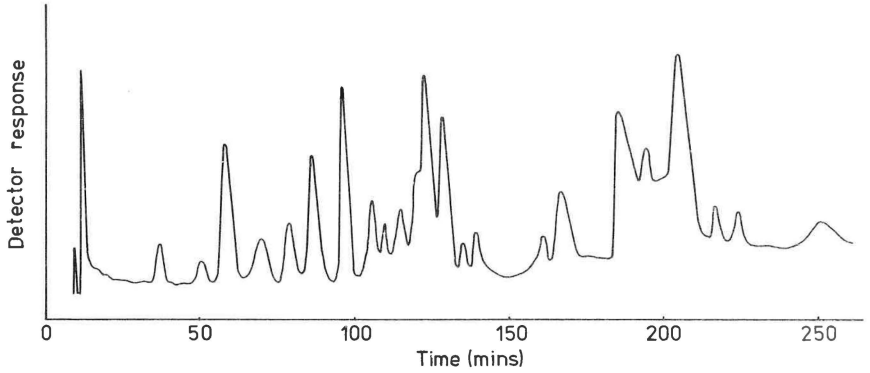


Figure 3.3 Gas–liquid chromatographic separation of low boiling products from the digest of a humic acid–sodium sulphide degradation reaction at 250 °C. (After Craggs, 1972)

conditions. However, labile bonds form only a small part of the structures in humic materials whose components are in the main linked by much stronger bonds, such as the C—C sigma bond, ether linkages, etc., cleaved only by highly energetic processes. These processes must be closely controlled to avoid degradation of the polymer to very simple but structurally meaningless products.

The most successful techniques used up to the present time for the degradation of humic substances yield large numbers of products. This is illustrated in Figure 3.3, which shows 24 compounds in the gas–liquid chromatography (g.l.c.) trace obtained by Craggs (1972) for methylated products in one fraction of a humic acid-sodium sulphide solution digest. Identification of such degradation products has been greatly accelerated by use of modern instrumental techniques such as preparative g.l.c., infrared, nuclear magnetic resonance (n.m.r.) and mass spectrometry. The direct coupling of gas chromatography and mass spectrometry (g.c.–m.s.), together with computer analysis of the output data, has provided a particularly powerful tool for identification of the many components in degradation products.

Frequently the products isolated from degradation reactions are regarded as the actual 'building blocks' of the humic polymers. Such interpretations can be false because, in many instances, the naturally occurring units are altered prior to release into the digests, and further alterations to the molecules can take place in the digests. For these reasons it is important to understand the reaction mechanisms which are likely to operate for the system being studied. This can be achieved by carrying out model studies with compounds of known structures which cleave, under the appropriate reaction conditions, to products of the types identified in the polymer degradation digest. It is important of course to use the same reagents and conditions for the polymer degradation and for model studies reactions.

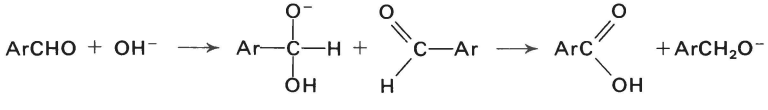
Little attention will be given here to mild hydrolytic procedures which attack only the more labile components of humic substances, or to drastic conditions, such as those oxidation reactions which degrade the polymers into structurally meaningless products such as carbon dioxide, water, and short-chain aliphatic carboxylic acids. Emphasis will be placed on chemical reagents and reaction conditions that give rise to products which could reasonably be assigned to structures in the polymer molecule. As will become clear, this is often difficult because many of the compounds identified could be derived from different precursors.

Degradation by Oxidative Processes

Oxidative processes involve the loss of electrons from the substrate leading to the rupture of carbon–hydrogen and carbon–carbon bonds. Such rupture is energetically demanding; the cracking of the carbon–hydrogen bond, for instance, requires 300–400 kJmole^{-1}, and most of this energy must be supplied by a simultaneous bond making between oxidant and substrate.

Bond cleavage can be homolytic, giving rise to radical species, or heterolytic leading to the formation of ionic structures. Homolytic splitting of the carbon–hydrogen bond results from initiation and attack by molecular oxygen, although peroxides and ionizing radiation are better initiators. Once initiated the reaction can, like other free radical reactions, be readily perpetuated. Where formation of the radical involves hydrogen atom abstraction the reaction is facilitated by resonance stabilization, as in the cases of allylic [$-\overset{|}{C}=\overset{|}{C}-\overset{|}{C}\bullet$] and benzylic [$Ar-\overset{H}{\overset{|}{C}}\bullet$, where Ar is the phenyl, C_6H_5 group] species. Oxidations of this type proceed more readily for hydrogen atoms on tertiary than on secondary and primary carbon atoms.

Proton and hydride (H^-) abstraction by chemical means are other well known mechanisms in oxidation reactions. Hydride extraction is well exemplified in base catalysed disproportionation reactions, such as the Cannizzaro reaction, for aldehydes lacking in α-hydrogen atoms. Benzenecarbaldehyde, for instance, when treated with base initially forms an addition product which then transfers a hydride ion to the aldehyde to give the reduction product (the alcoholate anion), and the residual oxidation product forms the acid. The reaction is completed by transfer of a proton from the acid to the alcoholate to form the alcohol and the acid anion.



(3.9)

Other oxidation mechanisms will be dealt with as they arise in different oxidative degradation reactions.

Oxidation by permanganate. Solutions of potassium permanganate ($KMnO_4$) are extensively used for the oxidation of organic chemicals. There is extensive literature dealing with various applications of permanganate oxidation techniques and the reader is referred to reviews by Stewart (1964, 1965) for general background information. Here we shall be concerned primarily with reactions of manganese (VII) and (VI) but it is well to remember that manganese can have oxidation numbers from +1 to +7.

The permanganate species, Mn(VII), is commonly used as the starting oxidant for the degradation of humic substances. It is quite stable in acid solution, and and in 30–50 % sulphuric acid it forms the highly reactive permanganic acid ($HMnO_4$), although this decomposes to a less reactive soluble manganese (IV) species in concentrated sulphuric acid. In general the reactivity of the different species varies inversely with charge, and their oxidative effects follow the order: $HMnO_4 > MnO_4^- > MnO_4^{2-} > MnO_4^{4-}$

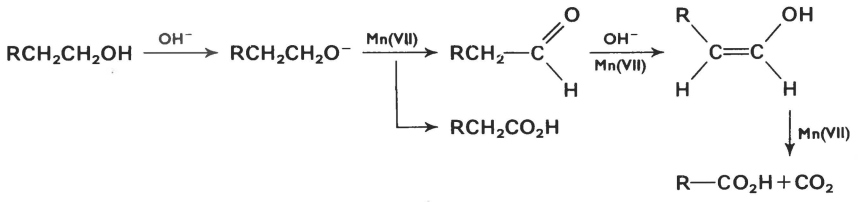
Because of the severe oxidation effects of $HMnO_4$ it is more usual to use a less drastic permanganate salt, e.g. $KMnO_4$ for the oxidation of humic substances. This gives a mildly alkaline solution which has the added advantage of being able to dissolve the humic substances to give a homogeneous reaction mixture. Under these conditions slow decomposition of permanganate occurs, and this is catalysed by MnO_2:

$$2MnO_4^- + H_2O \xrightarrow{MnO_2} 2MnO_2 + 2OH^- + \tfrac{3}{2}O_2 \qquad (3.10)$$

Since manganese dioxide is itself the final reaction product of oxidations by Mn(VII) it is necessary to use excess permanganate. In strongly alkaline solutions permanganate decomposes slowly to give manganate (VI) i.e. the MnO_4^{2-} species, which, although less reactive than permanganate, is still an effective oxidizing reagent.

The rates and the extents of oxidation of the different substrates are governed by their compositions, stereochemistry, the functional groups present, and especially by their solubilities in the media used. As a general rule anions are more readily oxidized than neutral molecules, which in turn are more readily oxidized than cations.

We shall now consider reactions of permanganate with some functional groups and linkages which are likely to be important in humic structures, beginning with alcoholic hydroxyls. Primary and secondary alcohols react more readily in basic than in neutral media because of the formation of the soluble anionic or alkoxide species. This will rapidly transfer a hydrogen atom or a hydride ion to the oxidant. Primary alcohols are oxidized to carboxylic

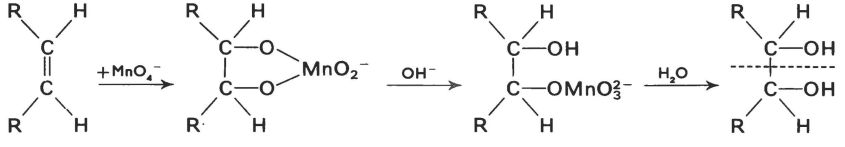


(3.11)

acids as shown in reaction scheme (3.11). Enolizable aldehydes and ketones (from secondary alcohols) can lose CO_2 to form the carboxylic acids with one carbon atom less than the original alcohol, as indicated by scheme (3.11). Tertiary alcohols resist oxidation, and are degraded only when very drastic conditions are employed.

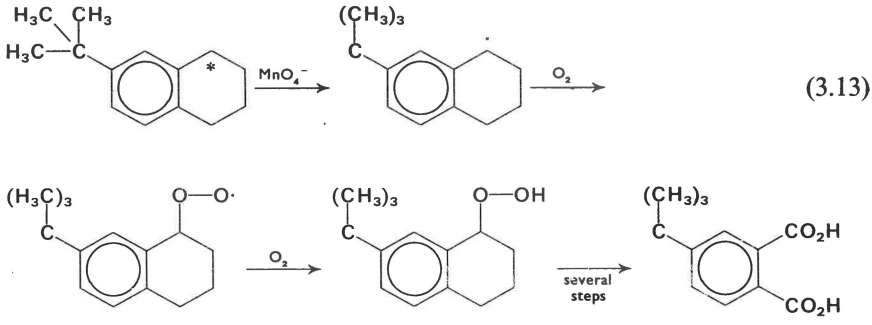
Phenols (benzenols) and aniline (benzenamine), in which the aromatic nucleus is activated by the electron-donating substituents, are degraded to carbon dioxide when treated with permanganate (Stewart, 1964, p. 75). However, methylation confers a good degree of resistance to breakdown, and the importance of this will be evident in the later considerations of alkaline permanganate degradations of humic substances.

Saturated aliphatic hydrocarbons are relatively resistant to oxidation by permanganate. Some oxidation is observed when the molecule is rendered more soluble by the presence of a carboxyl group. When the chain is branched the molecule is oxidized to tertiary hydroxyacids (Kenyon and Symons, 1953). Alkenes are significantly more susceptible to oxidation than alkanes. Glycol formation from alkenes in controlled reactions with permanganate is well known, and although the reaction takes place with aqueous permanganate it proceeds best under alkaline conditions. Cyclic manganese esters are first formed and the hydroxyl groups add *cis* (see Stewart, 1965, p. 42).



(3.12)

In hydrolysis of the Mn(V) ester to give the glycol in reaction scheme (3.12), Mn(VII) and Mn(IV) are formed by disproportionation. However, it should be remembered that further oxidation resulting in cleavage to carboxylic acids (as indicated by the broken line in a sequence (3.12)) always competes with glycol formation. Thus excess oxidant at higher temperatures will normally give rise



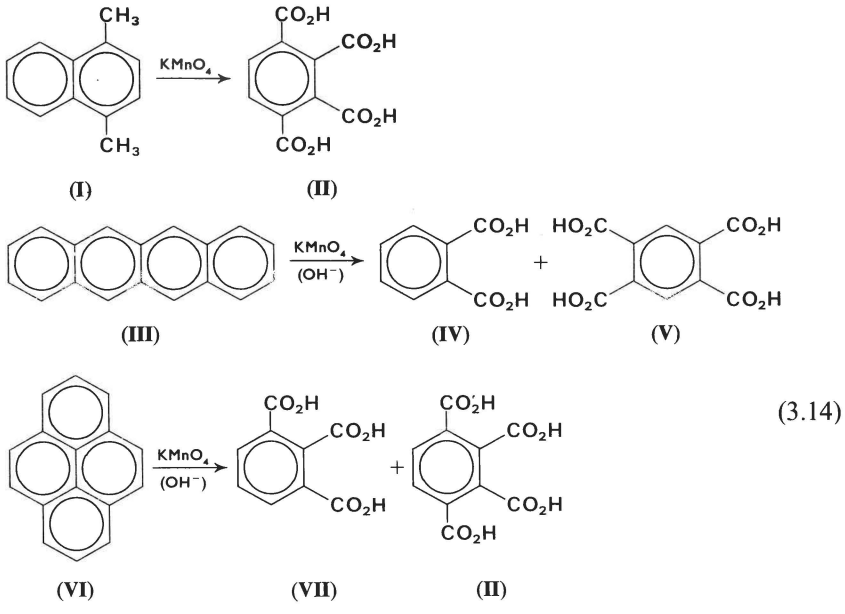
(3.13)

to cleavage. Alkynes are even more readily oxidized than alkenes and they form diketo, or more likely carboxylic acid cleavage products.

Permanganate oxidation of aromatic hydrocarbons is very relevant to degradation studies on humic substances. Reaction scheme (3.13), from Stewart (1964, p. 67) and Lee (1969, p. 37), illustrates products formed when an aromatic ring containing two different types of substituents is reacted with alkaline permanganate. This scheme shows that, in the absence of hydrogen on the α-carbon substituent, alkyl side chains resist degradation. Attack on the benzylic substituent (marked with an asterisk) is thought to cause expulsion of a hydrogen atom and the residual radical reacts with oxygen to give the peroxide, and degradation proceeds to the carboxylic acids. Some degradation of the aromatic ring occurs, particularly when excess permanganate is used, and, as previously indicated, complete degradation takes place when the ring has hydroxyl and amine substituents.

One or more rings of polycyclic aromatic structures are degraded in oxidations by permanganate. For instance 1,4-dimethylnaphthalene (**I**) gives 1,2,3,4-benzenetetracarboxylic acid (Bone *et al.*, 1935), naphthacene (**III**) gives 1,2-benzenedicarboxylic acid and 1,2,4,5-benzenetetracarboxylic acid (**V**), pyrene (**VI**) gives the 1,2,3-tricarboxylic acid (**VII**) and **II**. These reactions are summarized in reaction scheme (3.14) from Lee (1969, pp. 37–38).

Permanganate has been used for a long time for the oxidation of coal (Bone *et al.*, 1930; Bone and Himus, 1936; Ward, 1947; van Krevelen, 1961, pp. 225–231), lignin and wood (Lai and Sarkanen, 1971, p. 217; Chang and Allan, 1971, pp. 452–457). Piret *et al.* (1957) brought the permanganate degradation technique closer to the interests of soil chemists when they showed that peat degradation could yield 12 to 22% of aromatic polycarboxylic acids. More recently Schnitzer and his colleagues have extensively applied permanganate oxidation reactions to soil organic matter and humic substances. Their early work used potassium permanganate in dilute potassium hydroxide to oxidize the organic matter from the A and B_h horizons of an imperfectly drained podzol (Wright and Schnitzer, 1959b; Schnitzer and Wright, 1960a) and later to oxidize a humic acid preparation from the B_h horizon (Hansen and Schnitzer,



(3.14)

1966). Potassium hydroxide was omitted from subsequent work, and the humic substances were methylated with methyl iodide and silver oxide (Barton and Schnitzer, 1963), or dimethyl sulphate and anhydrous potassium carbonate in acetone (Briggs and Lawson, 1970), or with an excess of diazomethane, regarded by Schnitzer (1974a) as the best of the techniques.

The work of Matsuda and Schnitzer (1972) clearly illustrates the importance of premethylation. Substrate methylation had long been used to stabilize lignins prior to oxidation with permanganate (see Chang and Allan, 1971), because, as has been pointed out, the conversion to methoxybenzene derivatives could be expected to stabilize the phenolic constituents against degradation to meaningless products. Yields of oxidation products in the permanganate digests of humic acids from different soils were 160 to 250 % higher than those for the unmethylated materials after the polymers (1 g) had been refluxed for 8 hours in 250 cm^3 of *ca* 0.25M aqueous $KMnO_4$, pH 10 (Matsuda and Schnitzer, 1972). However, even methylated humic substances are degraded by prolonged reaction with excess permanganate (Schnitzer and Desjardins, 1970).

Figure 3.4 (assembled from selected data from Khan and Schnitzer, 1971 and 1972; Matsuda and Schnitzer, 1972; Schnitzer and Ortiz de Serra, 1973a Neyroud and Schnitzer, 1974a) includes compounds isolated from refluxed (8 hours) permanganate digests of humic and fulvic acids. These compounds were extracted in organic solvents, methylated, and fractionated and identified by g.l.c., g.c.–m.s. and microinfrared techniques. All of the carboxylic acids and phenols were identified as methyl esters and ethers, respectively. These

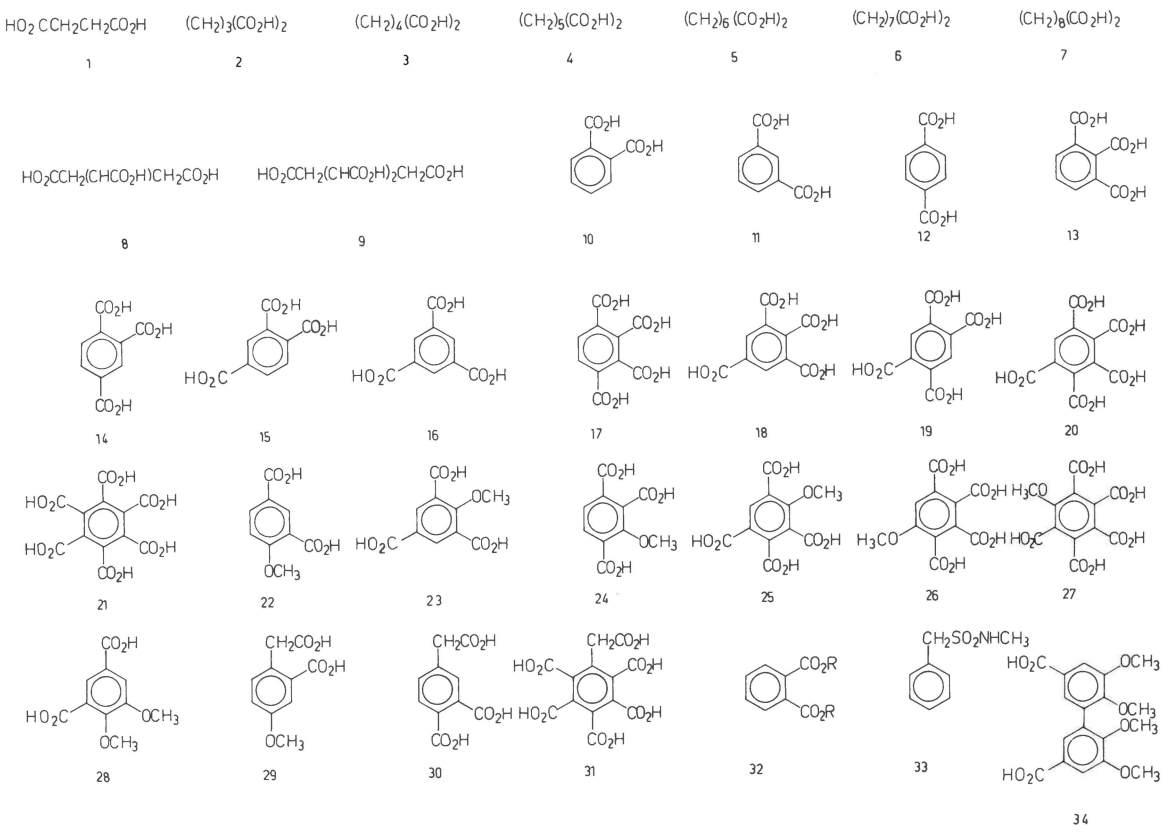


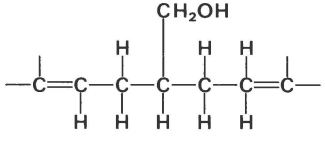
Figure 3.4 Products identified in the digests from permanganate oxidations of humic and fulvic acids

esters are represented by the free carboxylic acids. Some of the carboxyl groups originated from the humic substances, but many were formed during the degradation. By contrast few, if any, phenolic groups were produced during the degradation, and because it is impossible to say whether these were present as the ethers or hydroxyls in the polymer they are represented as the methyl ethers in Figure 3.4.

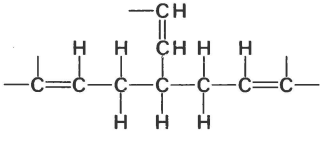
Aliphatic di-, tri-, and tetracarboxylic acids (compounds **1** to **9**) amounted to 20 % of the 0.194 g of digest products from the oxidation of humic acid (1 g) from the O horizon of the Armadale profile, and pentanedioic (glutaric, **2**), hexanedioic (adipic, **3**), heptanedioic (pimelic, **4**), and 1,2,3-propanetricarboxylic (**8**) acids were the most abundant of those present. Only octanedioic (suberic, **5**) and nonanedioic (azealic, **6**) acids were identified in the humic acid digests from the A_1 horizons of Japanese Volcanic and Diluvial soils (Matsuda and Schnitzer, 1972), and of the considerable amount of data inspected only the Armadale O horizon humic acid contained a substantial proportion of aliphatic di- and polycarboxylic acids in the permanganate digests. These were also present, but in relatively lesser abundance in the B_h humic acid of the same profile.

Unsaturated carbon to carbon bonds separated by three to nine single bonds could give rise to structures **1** to **7** in Figure 3.4. The appropriate mechanisms are outlined in reaction scheme (3.12), and the glycols formed would be expected to cleave to the dicarboxylic acids. The yields of products from alkene or alkyne functional groups would not be influenced by humic acid premethylation. Thus it is difficult to explain how the yields of di- and polycarboxylic aliphatic acids were less in the digest of unmethylated B_h humic acid than in the methylated sample.

A simple explanation for the occurrence in the digest of the aliphatic tri- and tetracarboxylic acids (compounds **8** and **9**) might involve carboxylic substituents in the chain, or the presence of hydroxymethyl side chains, or of branched chain olefinic structures, such as **VIII** and **IX**. These would degrade to the 1,2,3-propanetricarboxylic acid, and the mechanism for oxidation of alcohols is presented in scheme (3.11).



(VIII)

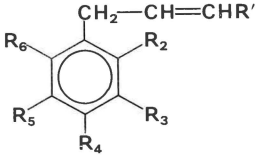


(IX)

All of the possible benzenecarboxylic acids, except benzoic acid, have been isolated and are shown in Figure 3.4 (structures **10–21**). These could have been formed by the oxidation of straight- and branched-chain substituents on a single benzene ring, or of aromatic and cyclic aliphatic structures of the type

indicated in scheme (3.13), or they could arise from fused aromatic structures. Some appropriate reactions are outlined in schemes (3.13 and 3.14).

The methoxy-substituted aromatic structures indicate the presence of phenols or of methoxybenzene derivatives in the humic structures. Compound **28**, 1,3-dicarboxy-4,5-dimethoxybenzene, would suggest that the carboxyl groups were derived from aliphatic straight- or branched-chain substituents, or from a cyclic aliphatic structure linked 1,3- to the benzene nucleus. One or both of the methoxy substituents could have been present as phenolic hydroxyls. Compounds **29**, **30**, and **31**, give further evidence for aliphatic side chains, and the α-substituted ethanoic acid derivatives could indicate the presence of unsaturation in one of the side chains, as represented in hypothetical structure **X**, where the R's could be hydrogens, aliphatic side chains, or a naphthalene structure in the case of **30** (3,4-dicarboxyphenyl) ethanoic acid, or a fused aromatic structure in the case of **31** (2,3,4,5,6-pentacarboxyphenyl) ethanoic acid. The 2-carboxy substituent in **29** (2-carboxy-4-methoxyphenyl) ethanoic acid was most likely derived from an aliphatic side chain, or from a cyclic aliphatic structure linked 1,2- to the aromatic nucleus, and containing an appropriately placed unsaturated bond.



(**X**)

A number of dialkylphthalate structures of the general formula **32** were isolated, and it is tempting to infer that these were derived from plastic contaminants. However, Khan and Schnitzer (1971) were aware of this, and their exhaustive tests eliminated the possibilities of contamination during the isolation, degradation, and the preparation of products for analysis. It is also unlikely that such compounds were formed by esterification when the permanganate digest was acidified because, if this were so, similar esters could also be expected for the other benzenecarboxylic acids, and such esters were not reported. In one unoxidized fulvic acid fraction, dicyclohexyl phthalate accounted for 74 % of the products identified, and the total dialkylphthalate content was 91 % of the 382.4 g identified (Khan and Schnitzer, 1971). Such structures in permanganate digests could arise from incomplete oxidation of phthalate structures in humic substances.

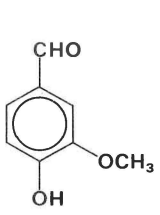
The presence of bis(3-carboxy-5,6-dimethoxy)biphenyl, compound **34** (or dehydrodiveratric acid) is thought to be indicative of the presence of some lignin type structures in humic substances. However, the possibility exists that compounds of this type could be formed by coupling through a free radical mechanism (Lai and Sarkanen, 1971) during the oxidative degradation process.

The full potential of permanganate oxidation techniques for structural studies on humic substances has not yet been realized. Considerable progress can undoubtedly be made from extended studies with aqueous solutions of $KMnO_4$. It will, however, be necessary to investigate the products formed by the use of different amounts of permanganate over varying conditions of time and temperature. For instance, studies with less than the theoretical amounts of permanganate required for complete oxidation would lead to enhanced decomposition of permanganate in the medium, as the result of catalysis by accumulating MnO_2 (reaction scheme 3.10). This would allow milder conditions to prevail in the digest and avoid over-oxidation of the most readily released compounds. By increasing the amounts of permanganate in separate experiments it would be possible to follow the alterations to these initially released compounds and to observe the release of new products in the digest. In this way it might be possible to obtain a better indication of the structures in the original polymer.

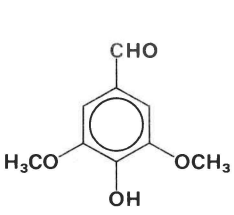
Use of excess permanganate, as developed by Schnitzer's group is particularly useful for comparisons between humic substances from different soil environments. One example of this has shown that podzol B_h humic substances have a higher enrichment of phenolic constituents than the same substances in the O horizon of the same soil profile (Matsuda and Schnitzer, 1972). This would substantiate podzolization theories which involve the movement of phenolic humic substances down the soil profile. The same work showed significant differences between the degradation products of Japanese volcanic soils and those of the podzol.

Permanganate oxidation can also give an indication of the degree of humification of soil organic constituents. High yields of degradation products are thought to be indicative of lower extents of humification. Lower yields might result from increased blocking of the 4-hydroxy substituents on the benzene ring (the formation of phenylether cross links would be an example) to give rise to more complex degradation products not yet identified. Some evidence of the effects of humification on such yields is provided by Khan and Schnitzer (1972). They showed that amounts of degradation products were lowered by cultivation practices; these presumably had caused oxidation of some of the humic substances. It is interesting to note that many of the products from the permanganate oxidation of fulvic acids were present in the non-degraded materials (Khan and Schnitzer, 1971; Ogner and Schnitzer, 1971). This might indicate that such products were released by air and/or biological oxidation processes.

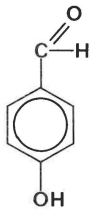
Oxidation by alkaline nitrobenzene. Following the pioneering work of Freudenberg *et al.* (1940), it has been established that when lignin is heated in the presence of 2M sodium hydroxide and nitrobenzene in a stainless steel pressure vessel for 2 to 2.5 hours at 170–180° C, vanillin (3-methoxy-4-hydroxy-benzenecarbaldehyde, **XI**), syringealdehyde (3,5-dimethoxy-4-hydroxybenzenecarbal-



(XI)



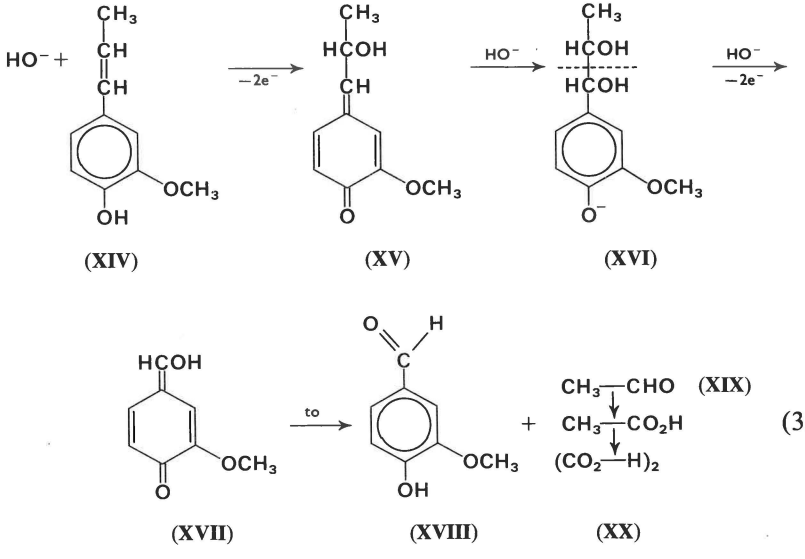
(XII)



(XIII)

dehyde, **XII**), and 4-hydrobenzenecarbaldehyde (**XIII**) or *para*-hydroxybenzaldehyde are the most abundant degradation products. Coniferous lignin degrades chiefly to **XI** with some of **XII** (Hurst and Burges, 1967). Woody dicotyledonous plants yield **XI** and **XII**, but **XI**, **XII**, and **XIII** are isolated from the degradation of the lignin of monocotyledonous plants.

Leopold (1952) has reviewed appropriate mechanisms and the oxidation of the model substance 2-methoxy-4-(1-propenyl)benzenol, or isoeugonol (compound **XIV**) is cited as an example in reaction sequence (3.15).



(XIV) (XV) (XVI) (XVII) (XVIII) (XIX) (XX)

(3.15)

In this sequence the oxidant, nitrobenzene acts as a two electron acceptor, and it undergoes stepwise reduction through nitrosobenzene and *N*-phenylhydroxylamine to aniline. Secondary condensation reactions can take place in alkaline media between the various products (Roberts and Casserio, 1965, pp. 869–870) to give rise to artefacts such as azoxybenzene, azobenzene, and 4-, and 2-hydroxyazobenzene, all of which have been isolated from lignin degradation

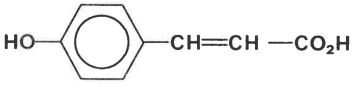
reactions in the alkaline nitrobenzene medium. This focuses attention on the need for caution where interpreting the origins of compounds in degradation reactions.

Reaction sequence (3:15) illustrates the importance of the quinonemethide type of structure as an intermediate in the oxidation of phenols under alkaline conditions. Under such conditions the phenolate anion of **XIV** is readily formed and this transfers two electrons to the nitrobenzene to allow the formation of the quinonemethide **XV** which then adds a hydroxide ion to give the aromatic structure **XVI**, the phenolate anion of 2-methyoxy-4(propane-1, 2-diol) benzenol. Further oxidation of **XVI** by the same mechanism would lead to cleavage along the broken line to yield **XVII** and ethanal (**XIX**) which further oxidizes to ethanedioic (**XX**) or oxalic acid. The quinonemethide **XVII**, would then rearrange to **XVIII**. The importance of the phenolic hydroxyl group and of the quinonemethide intermediate is emphasized by the fact that the methyl ether of **XIV**, which does not form the quinone methide structure, is not oxidized under the reaction conditions. The reaction will also not take place when the hydroxyl group is in the *meta* position.

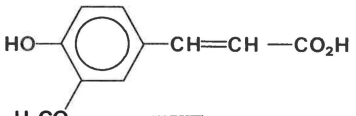
Gottlieb and Hendricks (1945) were first to apply the alkaline nitrobenzene technique to the degradation of soil organic matter. They failed to isolate 3,5-dimethoxy-4-hydroxybenzenecarbaldehyde (**XII**) and could only detect traces of the odour of 3-methoxy-4-hydroxybenzenecarbaldehyde (**XI**) in the digest of a New York muck soil. However, they isolated significant yields of ethanedioic acid (**XX**). Later excellent work by Morrison (1958, 1963), which illustrated the value of establishing the best reaction conditions, showed very good agreement between the total amounts of **XI**, **XII**, and **XIII** isolated from the alkaline nitrobenzene oxidation at 170 °C of a phragmites humic acid and of phragmites straw. By column and paper chromatography he identified and isolated 5.9 and 6.42 % of the carbon contents of the acid and straw, respectively, as the aldehydes **XI**, **XII**, and **XIII** and a further 1.95 and 1.06 % as the acid oxidation products of these aldehydes. At temperatures of 150 °C significant quantities of 3-(3-methoxy-4-hydroxyphenyl) propenoic acid (**XXII**) and of 3-(4-hydroxyphenyl) propenoic acid (**XXI**) were detected, but the amounts present were significantly less at 170 °C. These compounds would be expected to oxidize to **XI** and **XIII** in the reaction and their presence indicates a non-lignin source.

Other compounds detected included 1-(4-hydroxyphenyl)-1-ethanone (**XXIV**), 1-(3-methoxy-4-hydroxyphenyl)-1-ethanone (**XXV**), 1-(3,5-dimethoxy-4-hydroxyphenyl)-1-ethanone (**XXVI**), methanoic acid, ethanoic acid, and 9.8% of the peat carbon was lost as carbon dioxide.

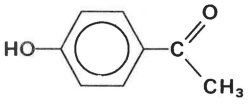
The work of Morrison and of Wildung *et al.* (1970) has shown that the yields of aldehydes decreased in the order, plant material > peat and eutro-amorphous peat (muck) > mineral soils. This suggests that the alkaline nitrobenzene degradation procedure could be used for the classification of soil organic matter. The more highly humified organic matter would be expected to provide low



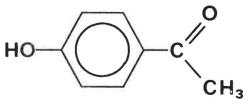
(XXI)



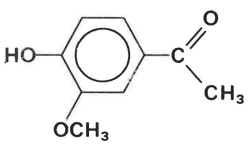
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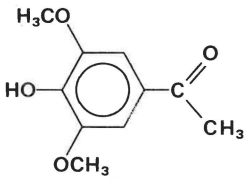
(XXIII)



(XXIV)



(XXV)

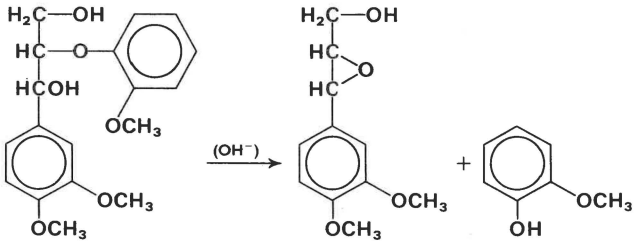


(XXVI)

yields of 4-hydroxybenzenecarbaldehyde derivatives, possibly because of the involvement of the 4-hydroxyl groups in ether or other linkages. Less humified material, with perhaps fewer cross-linkages or derivatives involving the 4-hydroxyl group, would give higher yields of benzenecarbaldehyde derivatives. It is tempting to infer that the less humified material might contain lignin residues which have only been partially modified.

In order to determine the full potential of the alkaline nitrobenzene procedure for the degradation of humic substances it will be necessary to degrade at different concentrations of alkali and nitrobenzene, at a range of temperatures, and in controlled atmospheres. The isolation for instance of **XXI**, and **XXII** at 150 °C is more significant than the carbaldehyde derivatives which would be formed at 170 °C. Model studies are required in order to make meaningful predictions of how such compounds could have been released, and of the types of functional groups from which they were released.

Oxidation by alkaline and alkaline cupric oxide solutions. Sodium hydroxide solutions at elevated temperatures are used for lignin degradation (Wallis, 1971) and it is therefore not surprising that similar procedures have been used extensively to degrade humic substances. Studies with models representing structural units in lignins have shown how ether linkages in the aliphatic side chain of phenylpropane structures can be broken. Compound **XXVII**, 1-(3,4-dimethoxyphenyl)-2-(2 methoxyphenoxy)propan-1,3-diol, when reacted for 2 hours at 170 °C with 2M sodium hydroxide cleaved (Gierer and Norén, 1962)



(XXVII) **(XXVIII)** **(XXIX)** (3.16)

as indicated in reaction scheme (3.16). This is known as β-ether cleavage, and it requires the presence of a hydroxyl group on the α- or γ-carbons of the aliphatic side chain. The scheme shows that the phenoxy substituent is cleaved in base to the 2-methoxybenzenol (**XXIX**) or guaicol, and that an epoxide structure (**XXVIII**) is formed which hydrolyses to the triol. When the *para*-substituent in **XXVII** is hydroxyl the epoxide structure can still be formed which can hydrolyse to the triol as before. However, the hydroxyl substituent also allows the formation of a quinone methide, and the elimination of water and methanal from the side chain.

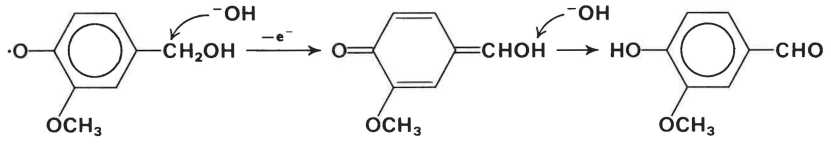
Ethers, in which the oxygen linkage is attached to the carbon of the propane side-chain alpha to the benzene ring (α-ethers) are also cleaved, but the studies of Gierer and Norén (1962) indicate that such cleavages require the presence of a hydroxyl group *para* to the propyl side chain. This emphasizes the importance of the quinonemethide intermediate in the reaction.

Alkaline solutions of cupric, mercuric, silver, and cobalt oxides have been used to degrade lignin to aromatic aldehydes and aromatic carboxylic acids as the major products. The acid to aldehyde product ratio is governed by the oxidizing potential of the oxidant. Thus silver oxide, which has the highest oxidizing potential of the four, produces mainly acids, mercuric oxide gives a mixture of acids and aldehydes, and cupric oxide, a relatively weak oxidant, yields mainly aromatic aldehydes (Chang and Allan, 1971). Of these oxidants only alkaline cupric oxide has been used extensively so far for the degradation of humic substances.

Nigh (1973) has reviewed the type of complexes formed by copper(II). He also presented a comprehensive list of oxidation products and provided mechanisms for reactions carried out for the most part in non-aqueous systems. However, this list is only of limited use for studies with humic materials where degradations generally involve aqueous media. Extensive model studies of substrate Cu(II) interactions will be required in order to appreciate the mechanisms of alkaline cupric oxide degradations of lignins and humic substances.

There are some similarities between alkaline nitrobenzene and alkaline cupric oxide degradations; the most important is that both yield aldehydes in

the digest. Copper oxide has a significant advantage because artefacts, such as those found in the nitrobenzene system, are not formed. They differ also in the fact that cupric oxide is a one-electron transfer oxidant. In their review on lignin oxidation Chang and Allan (1971, pp. 444–446) suggest that the reaction is initiated by extraction of an electron to give a phenoxy radical. A second electron is then rapidly transferred to another cupric oxide molecule preventing coupling and allowing the formation of the quinonemethide structure as indicated in reaction scheme (3.17).



(XI) (3.17)

The high temperature and excess cupric oxide used in such reactions increases the rate of electron transfer but does not affect the coupling reaction, which requires no activation energy. The importance of using excess copper oxide is evident from the fact that the highest yield of 3-methoxy-4-hydroxybenzene-carbaldehyde (**XI**) was obtained when 13.5 moles of reagent was used per lignin building unit (referenced by Chang and Allan, 1971).

Figure 3.5 (which also refers back to compounds in Figure 3.4) illustrates the numbers and the types of products which have been separated after humic and fulvic acids were degraded in alkaline and in alkaline cupric oxide solutions. The list is compiled from data from Greene and Steelink (1962), Jakab *et al.* (1963), Schnitzer and Ortiz de Serra (1973a), Schnitzer (1974b), Neyroud and Schnitzer (1974a; 1974b, 1975), and Griffith and Schnitzer (1976). Degradation products reported by Greene and Steelink and by Jakab *et al.* were separated by paper chromatography, and those from Schnitzer's laboratories were identified by g.c.–m.s. and micro-infrared after they had been extracted with solvent from the digest and methylated with diazomethane. Again phenolic hydroxyls are represented as the methyl ether derivatives, whereas the identified methyl esters are presented as the appropriate carboxylic acids.

Comparisons, where possible, of yields of solvent-extractable materials have shown that hydrolysis in 2M NaOH produces at least as much soluble material as oxidation by the CuO–NaOH system (Neyroud and Schnitzer, 1974a). However, a higher proportion of the products from the oxidation reactions have been identified indicating that the oxidation produces simpler compounds, or more advanced degradation, than does the hydrolysis. Some aliphatic dicarboxylic (compounds **1**, **2**, **3**, **6**), tricarboxylic (**8**), and tetracarboxylic (**9**) acids were released by base from humic and fulvic acids, as was observed for permanganate oxidation. The release of aliphatic hydrocarbons (**35**), and of fatty acids (**36**; up to 10% of the weight of the humic substances) was, however,

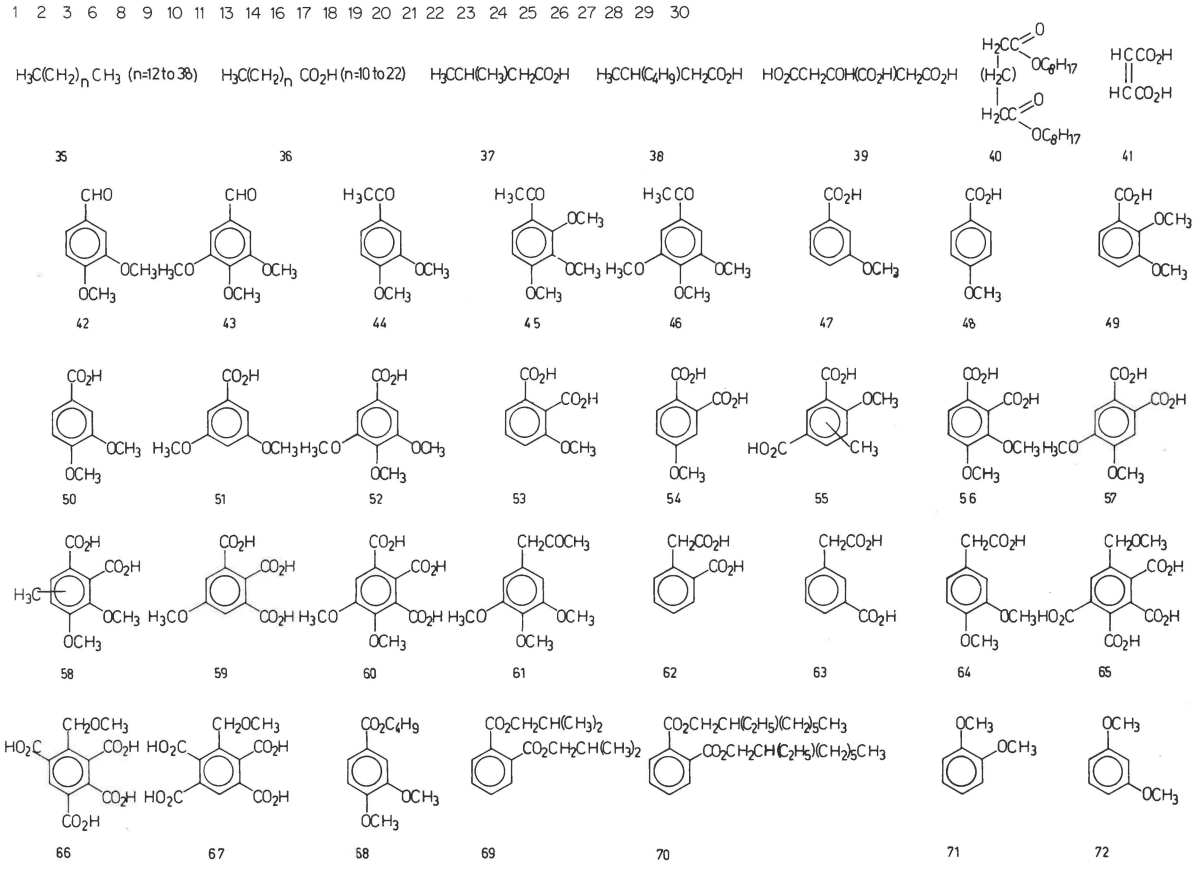
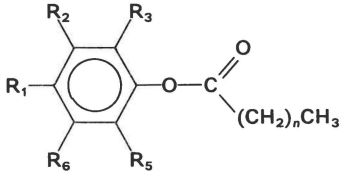


Figure 3.5 Products identified in the digests from alkaline cupric oxide degradations of humic and fulvic acids

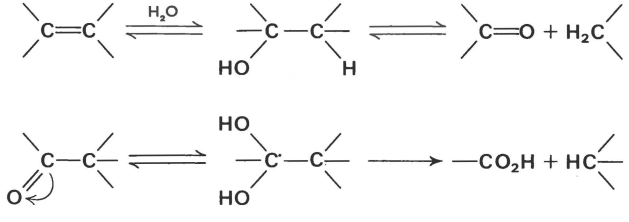
surprising. Ultrasonication, followed by hydrolysis in water at 170 °C gave the maximum yield (1.7 % of the total weight) of alkanes from fulvic acids, but hydrolysis in 2M NaOH at 170 °C for 3 hours was required to obtain the high fatty acid yields reported (Schnitzer and Neyroud, 1975). It is difficult to explain, without additional experimental evidence, why the alkanes were not released in the basic medium. The authors observed that the majority of the alkanes were in the C_{18} to C_{36} range and that they had an odd to even carbon ratio of 1.0 which might indicate a microbial origin.

Considerations of the phenolic and fatty acid saponification products suggested to Schnitzer and Neyroud that these were largely held as phenolic esters as represented in structure **XXX**, where the R's represent hydrogen, aliphatic or aromatic substituents, carboxyl groups, or hydroxyl or ether derivatives.



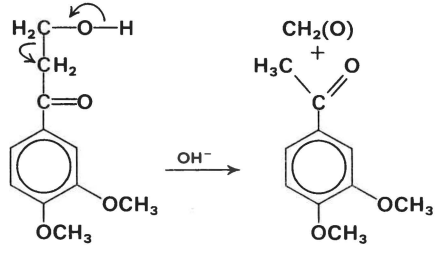
(XXX)

Reaction scheme (3.18) outlines mechanisms other than saponification which, according to Shemyakin and Shchukina (1956), would release acids and also hydrocarbons into the basic digests from alcoholic, ketonic, and alkene substituents in aliphatic structures. The reader can readily deduce how the positioning of such functional groups along the hydrocarbon components of humic substances would give rise to aliphatic dicarboxylic acids, monocarboxylic acids, and alkanes. Should compound **38** result from such release mechanisms it would indicate the presence of branched structures in the chain.



(3.18)

Benzenecarbaldehyde derivatives **42** and **43**, when present in CuO–NaOH digests, might suggest that they had origins in lignin type structures. The presence of ketones **44**, 1-(3,4-dimethoxyphenyl)-1-ethanone, **45** and **46** would substantiate this hypothesis because these could arise from the cleavage of



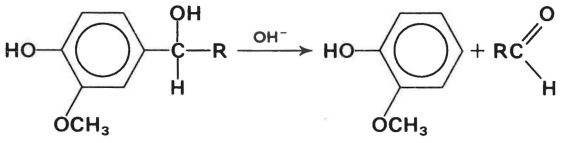
(XXXI) (XXXII) (3.19)

β-ethers of the type shown in reaction scheme (3.16). Appropriate mechanisms for ketone formation are outlined (from Wallis, 1971) in reaction scheme (3.19).

Schnitzer and Ortiz de Serra (1973*a*) found that compounds **42**, **43**, **44** and **46** amounted to 26% of the weight of the structures identified in the CuO–NaOH digest of an unmethylated humic acid from the A_1 horizon of a Brunizem soil. The acids **50** and **52**, which might have been formed from more advanced oxidation of the same parent materials, amounted to an additional 14% of the identified mass. When the humic acids were methylated before degradation the values were 23% for aldehydes plus ketones and 20% for the related acids. Compound **45**, 1-(2,3,4-trimethoxyphenyl)-1-ethanone was also present in substantial yields in the degradation products of the unmethylated materials. These results were unusual because aldehyde and ketone derivatives were not detected in many of the digests of other humic substances although the appropriate acids were. Generally, when compounds **42**, and **43** (aldehydes), and **50** and **52** (acids) were present in the same digest the acids were significantly more abundant (Schnitzer, 1974*b*; Griffith and Schnitzer, 1976).

The information in Figures 3.4 and 3.5 indicates that the benzenecarboxylic acids released by permanganate oxidation are also released by NaOH hydrolysis and by CuO–NaOH oxidation. Also it will be obvious from comparisons between the compounds in the two figures that a greater variety of methoxybenzene- or of hydroxybenzenecarboxylic acids are preserved in the basic solutions than in the permanganate digests. The methoxy substituents detected were either present as such in the parent molecule, or more likely were derived from a combination of methoxy and of hydroxy substituents, phenolic esters, and less likely from β-ether structures. Jakab *et al.* (1963) have isolated 1,2- and 1,3-dihydroxybenzenes (shown as the methyl ethers in structures **71** and **72**) and we have referred to mechanisms, such as those in reaction scheme (3.16) which would release the methoxybenzenol derivatives from β-ether structures. Wallis (1971) has indicated that structure type **XXXIII** could also cleave in alkaline conditions to **XXXIV** and the aldehyde in reaction scheme (3.20).

It is almost certain that some carboxyl groups in the various benzenecarboxylic acid compounds were present as substituents on the aromatic structures of the parent polymers. However, it is probable that many or most of these were



(3.20)

(XXXIII) (XXXIV)

generated under the alkaline oxidative conditions from side chains which contained appropriate alkene (or alkyne), hydroxyl, keto, and other substituents which would give reactions of the types outlined in scheme (3.18).

In the cases of degradations with permanganate reference was made to the possibility that fused aromatic structures gave rise to some of the polycarboxylic acids identified. Such fused structures would be resistant to degradations in 2M NaOH media; yet none were detected. Therefore it is likely that the polycarboxylic acids in Figure 3.4, which could have been formed from fused aromatic structures, were derived from another source.

The presence of phenylethanoic acid structures (**62** to **64**) provides direct evidence for aliphatic substituents in the aromatic nuclei, and mechanisms already discussed, particularly those in scheme (3.16), would allow such structures to be formed from appropriate functional groups located on carbon atoms β- to the aromatic nucleus. Reaction scheme (3.18) shows how the generation of a carbonyl group on the β-carbon could give rise to the methyl substituents in compounds **55** and **58**.

Benzylmethyl ethers **65**, **66** and **67** would not be expected to be formed as the result of methylation (with diazomethane) of the appropriate primary alcohol structures, but they could result from methylation of enols followed by cleavage reactions. It is, of course, possible that the ether structure existed in the parent macromolecular compounds. A number of aromatic esters were also present in the digests, but these should have been hydrolysed in the alkaline medium. Therefore it can be assumed that they formed on acidification of the mixtures. Also sulphonic acid and sulphonamide derivatives were isolated. Some of these might have been artefacts from reagents used to generate diazomethane, but others could indicate microbial origins (Neyroud and Schnitzer, 1975).

It can be concluded, particularly from the studies by Schnitzer and his colleagues, that alkaline hydrolysis and alkaline cupric oxide oxidation have provided useful techniques for the degradation of humic substances. Unmethylated humic and fulvic acids, when degraded in CuO–NaOH solution, yield ethylacetate soluble compounds which, after methylation, amount to 10–50 per cent of the weight of starting material. Although product identification has varied from 10 to 90 per cent of the weights of solvent soluble materials, it is clear that a broader range of compounds survive this reaction than they do permanganate oxidations. In particular CuO–NaOH preserves more of the aliphatic and phenolic structures than does $KMnO_4$, and it therefore allows

better comparisons to be made between humic substances from different environments.

Oxidation by nitric acid. Nitric acid has been extensively applied in studies on lignins (Dence, 1971) and coal (Van Krevelen, 1961, pp. 220–224), but it has only been used to degrade soil humic substances (Schnitzer and Wright, 1960b; Scheffer and Kickuth, 1961a; 1961b; Hayashi and Nagai, 1961; Hansen and Schnitzer, 1967). This limited application may be partly attributed to earlier difficulties in quantitatively isolating the nitro derivatives formed, and to the problems of assigning origins in the polymers for these products. Digestions in nitric acid can give rise to a variety of aromatic substitution and replacement reactions in addition to oxidation processes.

Nitronium (NO_2^+) ions are usually generated for aromatic substitution reactions from concentrated nitric and sulphuric acids. They are present only in concentrations of about 4% as the result of self-dehydration reactions of concentrated nitric acid:

$$HONO_2 + H^+ \rightleftharpoons H_2ONO_2^+ \rightleftharpoons H_2O + NO_2^+$$

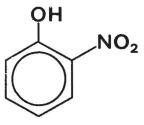
Thus concentrations of the nitronium species will be very low for the acid concentrations (e.g. the 2M and 7.5M nitric acid solutions applied by Hansen and Schnitzer, 1967) used to degrade humic substances. Therefore nitrosation (by NO^+) probably initiates the nitration reaction, as has been observed for lignins (Dence, 1971).

Nitroaromatic compounds can also arise from the electrophilic displacement by nitro groups of some ring substituents *ortho* and *para* to activating functional groups. Displacement will take place if the departing ionic species can be stabilized. For instance the leaving $^+CH_2OH$ group is stabilized by loss of a proton and rearrangement of the residue to methanal. The methyl ether derivative will not be stabilized in this way and will not be displaced. Neither will primary alkyl groups because their carbonium ions cannot be stabilized by simple proton transfer, but carboxyl and keto groups attached to the aromatic nucleus are readily displaced.

Nitric acid cleaves alkyl–aryl ethers by a solvolytic process and liberates the appropriate phenol and alcohol (Dence, 1971). The phenols are then nitrated, but are also highly susceptible to oxidative degradation by nitric acid to non-aromatic structures. Oxidation of polyhydroxy aromatic compounds goes through the corresponding *ortho* or *para* quinone intermediates and these can participate in addition reactions typical of conjugated dienone structures, or they can cleave to dicarboxylic acids and CO_2.

Yokakawa *et al.* (1962) when studying the degradation of coal humic acids investigated the degradation of a number of model aromatic compounds in boiling M HNO_3. Catechol and hydroquinone (2- and 4-hydroxybenzenol, respectively) lost 22% and 44% of their carbon contents as ethanedioic acid and CO_2, and some nitration of the aromatic structures was observed. Cinnamic,

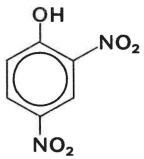
or 3-phenyl-propenoic acid, was resistant to degradation in the medium, but its phenol analogues were not. Methylation decreased the loss of CO_2 from the humic acid from 30 to 17 %, although the authors were not satisfied that methylation had been complete.



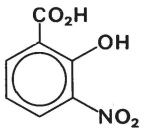
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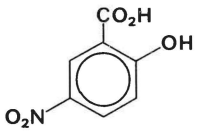
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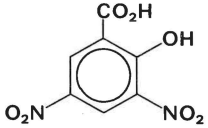
(XXXVII)



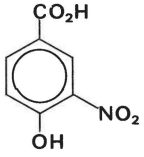
(XXXVIII)



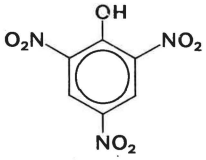
(IXL)



(XL)



(XLI)



(XLII)

On the basis of R_f values from paper chromatography, and n.m.r. data, Hansen and Schnitzer (1967) identified the following nitrobenzene derivatives in the benzene soluble fraction of the 2M HNO_3 digest: small amounts of 2- and of 4-nitrobenzenols (**XXXV**) and (**XXXVI**); possibly some 2,4-dinitrobenzenol (**XXXVII**); 2-hydroxy-3-nitrobenzenecarboxylic acid (**XXXVIII**); 2-hydroxy-5-nitrobenzenecarboxylic acid (**IXL**); 2-hydroxy-3,5-dinitrobenzenecarboxylic acid (**XL**); and 3-nitro-4-hydroxybenzenecarboxylic acid (**XLI**). Only picric acid, or 2,4,6-trinitrobenzenol (**XLII**) was present in the benzene extract of the 7.5M nitric acid digest. Nitroaromatic structures accounted for 2.1 % of the digests in 2M acid, and picric acid composed 5.30 % of that in the 7.5M acid (5.93 and 9.38 % of the weights of starting materials were identified as organic structures in extracts from the 2M and 7.5M acid digests, respectively). The nitrophenols shown can be explained by substitutions *ortho* and *para* to the hydroxyl groups and do not require the electrophilic displacement mechanism to be invoked.

Twelve benzenecarboxylic acids (compounds **10–21** in Figure 3.4) were detected, and amounted to 3.8 % of the starting material in the 7.5M digest. These were probably formed by oxidation of carbon side chains. There was evidence for small amounts of pentanedioic (**2**), hexanedioic (**3**), and heptanedioic (**4**) acids in the 7.5M digests. The hydroxybenzenecarboxylic acids identified, or tentatively identified were: 2-, 3-, and 4-hydroxybenzenecarboxylic acids and 2,4-, 3,5-, and possibly 3,4-dihydroxybenzenecarboxylic acids, and these amounted to about 10 % and 2.2 % of the compounds identified in the 2M and

7.5M digests, respectively, which suggests that such compounds were degraded at the higher acid concentrations. Indeed the evolution of CO_2 from digests (Yokakawa *et al.*, 1962) shows that nitric acid treatment causes extensive decomposition of the degradation products, but the technique may have potential when the reaction is preceded by adequate methylation.

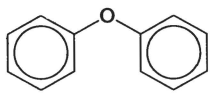
Degradation by reductive processes

Reactions which add hydrogen to a compound to form new covalent linkages are generally called reduction reactions. The most useful reducing agents in organic chemistry are hydrogen, generally used with a solid catalyst, metals such as zinc and sodium which transfer electrons to the substrate, and metal hydrides which transfer hydride (H^-) anions. In the transfer process the metals are oxidized to their cationic species. Hydride transfer reactions can also take place between certain molecules in basic media to yield oxidation and reduction products, as was described for the Cannizzaro reaction in reaction scheme (3.9). We will be mainly concerned here with reductions by active metals.

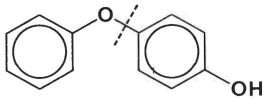
Reduction with sodium amalgam. Burges *et al.* (1964) introduced the sodium amalgam (Na/Hg) technique for the degradation of humic acids and isolated yields of 30–35 % ether-soluble products from the digests. These products, composed for the most part of mixtures of phenols and phenolic carboxylic acids, were readily oxidized to a dark residue on standing. Since that time the technique has been used by a number of workers (Mendez and Stevenson, 1966; Stevenson and Mendez, 1967; Dormaar, 1969; Schnitzer and Ortiz de Serra, 1973a, 1973b; Martin *et al.*, 1974) who obtained lower yields of ether or solvent soluble materials. Amongst the many compounds identified, the most readily released were 3-methoxy-4-hydroxybenzenecarboxylic acid (vanillic acid **93**) and 3,5-dimethoxy-4-hydroxybenzenecarboxylic acid (syringic acid **94**), and these were common to all digests despite differences in the ratios of reagents and reactants used.

Piper and Posner (1972) studied the mechanisms of reaction, investigated the optimum conditions for Na/Hg degradations, and established the importance of the ratio of reactant to reagent and of the time of reaction. Their work showed that the same products were released from hydrolysed and unhydrolysed substrate, and illustrated the importance of using finely ground amalgam (prepared according to Vogel, 1956). It also showed the amounts of monomers released increased with time during 30 hours of reaction. The results suggested that under the experimental conditions employed, best results were obtained by using 25 mg of the more highly oxidized humic acids (such as those extracted with pyrophosphate) and 50 mg of less oxidized material (e.g. alkali extracted humic acid) in an alkaline medium (15 cm^3 of 0.5M NaOH) containing 30 g of 5% sodium amalgam. Whilst the reaction conditions used by Burges *et al.*

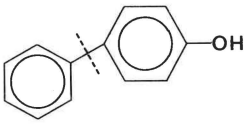
(1964) met these requirements, those used by other workers did not, and this might account for their lower yields.



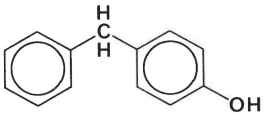
(XLIII)



(XLIV)



(XLV)



(XLVI)

Degradations on model compounds by Piper and Posner (1972) give indications about some relevant linkages which could be cleaved in Na/Hg digests. Although the mechanisms involved are not known, degradation is thought to result from attack by atomic hydrogen (H•). These atoms combine readily to form hydrogen gas (H_2), and this might account for the necessity to use excess amalgam. The hydrogen acts as an electrophile, as indicated by the products released during degradation of compounds **XLIII** to **XLVI**. Compound **XLIII** gave benzene and benzenol, **XLIV** gave equal amounts of benzenol because the electron density on the *para*-carbon is increased by the activating effect of the 4-hydroxy-group, and this would favour cleavage as indicated by the broken line. Again a similar activating effect caused **XLV** to cleave to equal amounts of benzene and benzenol, but **XLVI** did not cleave because excess electron density cannot reside on the bridging methylene carbon.

Figure 3.6 summarizes the products (Burges *et al.*, 1964; Piper and Posner, 1972; Dormaar, 1969; Schnitzer and Ortiz de Serra, 1973b; and Martin *et al.*, 1974) which have been identified in humic acid-Na/Hg digests. Model studies by Piper and Posner (1972) indicated that compounds **73**, **74**, **87**, **92**, **93**, **94**, and 4-hydroxybenzenol (hydroquinone) did not degrade when subjected to their reaction conditions, and this suggested that the identified phenolic structures in the humic acid digests could reasonably be assigned to structures in the polymer. Later, however, Martin *et al.* (1974) showed that varying extents of degradations occurred in their digests in the cases of compounds **75**, **76**, **88**, **89**, **91**, benzoquinone, 2-hydroxy-6-methylbenzenecarboxylic acid, and several hydroxynaphthenic acids. Decarboxylation was the principal reaction observed in the cases of the dihydroxybenzenecarboxylic acids, but the partial destruction of the trihydroxybenzene structures indicates that the yields reported for such compounds in humic acid digests were not representative of the amounts released as carboxylic acid derivatives. Variations in reaction conditions (e.g.

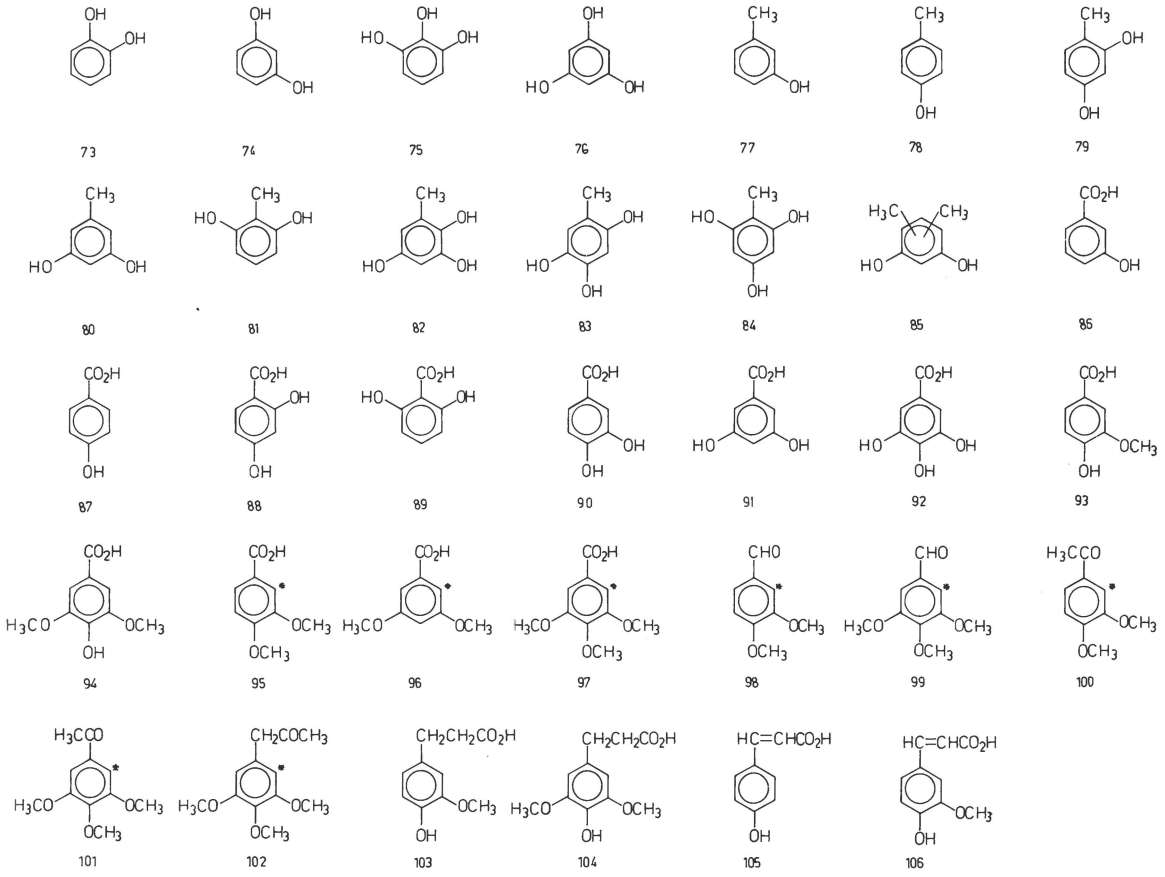
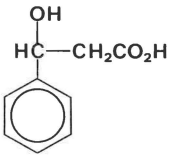


Figure 3.6 Products identified in the digests from sodium amalgam degradations of humic acids

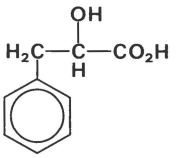
the amounts of reductant used, and the maintenance of anaerobic conditions in the alkaline media) might account for some of the differences in the apparent stabilities of the compounds examined.

On the basis of the studies of degradations of model substances by Martin *et al.* (1974) we cannot be certain that all of the non-carboxylic phenolic structures in Figure 3.6 represent primary structures in the humic acid polymers. Also, the fact that none of the phenolic carboxylic acids listed have a methyl group attached to the aromatic ring suggests that such structural types did not exist in the original polymer, or (if they did) were decarboxylated when released into the digest. It would appear that the various cleaved structures were linked by aromatic ether linkages, or were present as biphenyl structures where activating (hydroxy or methyl) substituents were *ortho* or/and *para* to the linking bond. The model studies, and the compounds identified would suggest also that methylene and longer aliphatic bridging structures were not cleaved.

Detection of the phenolic compounds and their quantitative estimations generally used two-dimensional chromatography and co-chromatography with standards. Compounds marked with an asterisk in Figure 3.6 (taken from the data of Schnitzer and Oritz de Serra, 1973b), were methylated prior to g.c.–m.s. and micro-infrared analysis, and it is impossible to state which of the structures originally had free hydroxyls. However, it is highly likely that the distribution of —OH and —OCH_3 substituents were the same as for the other similar compounds in the Figure 3.6. It is interesting to note that these workers isolated the aldehyde and keto structures **98**, **99**, **100**, **101** and **102**, and this is good evidence for the presence of the carbonyl group in humic substances.



(XLVII)



(XLVIII)

The side chain double bonds of the substituted propenoic acids **105** and **106** would be expected to rapidly hydrogenate, and Piper and Posner (1972) suggested that these might arise by dehydration of appropriate hydroxy derivatives **XLVII** and **XLVIII** during the drying of the ether extracts.

Despite the reducing conditions of the reaction many of the compounds in Figure 3.6 contain the carboxyl group. This provides strong evidence that this functional group, as well as hydroxyl, methoxyl, and carbonyl are present as substituents on aromatic nuclei of humic substances. This inference could not be made so strongly for carbonyl and carboxyl groups solely on the basis of oxidative degradation reactions.

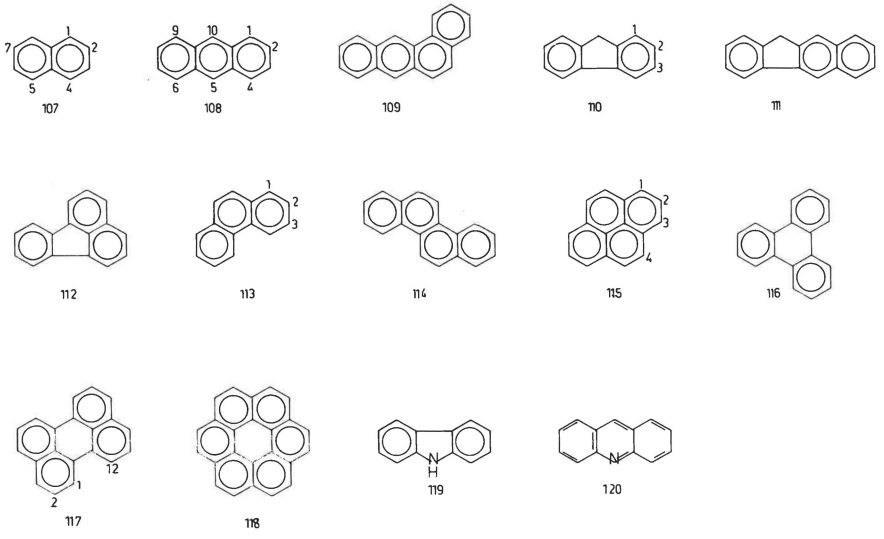


Figure 3.7 Products identified in the digests from degradations of humic materials in zinc-dust distillation and fusion reactions

Degradation by zinc-dust distillations and fusions. The zinc-dust distillation of humic substances has been described by Cheshire *et al.* (1967, 1968) and by Hansen and Schnitzer (1969a and 1969b). The latter authors (1969b) have also used zinc-dust fusion, and the products which have been obtained by both procedures are summarized in Figure 3.7. For the distillation process Cheshire *et al.* used 0.5 g quantities of acid (6M HCl) boiled humic acids mixed with 30 g purified zinc dust, and they placed an additional 10 g of the dust at the exit port of the pyrex tube used for the reaction. Distillation was carried out in a stream of H_2 gas at 500–550 °C. For fusion Hansen and Schnitzer combined 0.5 g of substrate with 3 g of Zn dust, 5 g $ZnCl_2$ and 1 g NaCl and heated at a temperature of 300 °C.

As Figure 3.7 shows the products isolated were composed of fused aromatic structures. The listed compounds are naphthalene (**107**), anthracene (**108**), 1,2-benzanthracene (**109**), fluorene (**110**), 2,3-benzofluorene (**111**), fluoranthene (**112**), phenanthrene (**113**), chrysene (**114**), pyrene (**115**), triphenylene (**116**), perylene **117**), coronene (**118**), carbazole (**119**), and acridine (**120**). In addition a number of derivatives of these were identified which include methylated naphthalenes (1-methylnaphthalene, 2-methylnaphthalene, and 1,2,7-trimethylnaphthalene), methylated anthracenes (1-methylanthracene and 9-methylanthracene), methylated phenanthrenes (2-methylphenanthrene and 3-methylphenanthrene), methylated pyrenes (1-methylpyrene and 4-methylpyrene), benzpyrenes (1,2-benzopyrene and 3,4-benzopyrene) and naphtho-(2′,3′:1,2)-pyrene, 1,12-benzoperylene, and homologues of carbazole and of acridine.

The yields of products from zinc-dust distillation reactions are invariably very low, often less than 1 %, and the recovery of 3 % of starting material as a pale yellow oil by Cheshire *et al.* (1968) was considered to be good. Hansen and Schnitzer obtained yields of the order of 0.6–0.7 % for humic acid, whilst those for fulvic acid were somewhat lower. Assuming a 10 % recovery of products and calculating on a functional group-free basis, they postulated that fused aromatic structures could account for 25 % and 12 % of the components in fulvic and humic acids, respectively.

Based on their data for zinc-dust distillation experiments, Cheshire *et al.* (1967) and Haworth (1971) proposed that humic substances are built around a polynuclear aromatic 'core'. Combining this with other data from hydrolysis, oxidation, reduction, and electron spin resonance (e.s.r.) they suggested that polysaccharides, simple phenols, proteins or peptides, and metals were attached to this 'core'.

The results from such drastic procedures must be interpreted with caution. In their later paper Cheshire *et al.* (1968) showed that 3,4- and 3,5-dihydroxybenzoic acids, furfural (from dehydration of pentose sugars), and quinone polymers gave polycyclic aromatic structures from zinc-dust distillation at 500–550 °C. At 400 °C only small yields of anthracene were obtained from the hydroxybenzenes, and no aromatic structures were detected in the distillates from furfural and polymers of *ortho*- and of *para*-benzoquinone. In addition, the same products were isolated in the same proportions from the distillations at the higher and lower temperatures and it was concluded that the products identified were largely released from the humic materials.

We conclude that judgement cannot yet be passed on the usefulness of zinc-dust distillation and fusion techniques for work with humic substances. An obvious experiment, which could help decide whether or not the fused aromatic structures detected were 'core' products or artefacts, would be to subject compounds released by other reductive techniques (such as the sodium amalgam method), and the undegraded residue to zinc-dust distillation at 400 °C, and to compare the yields of fused aromatic structures from both substrates.

Degradation with phosphorus and hydriodic acid. Hydriodic acid (HI) is a standard reagent for cleaving ether linkages, and red phosphorus provides reducing conditions to hydrogenate aromatic nuclei. A volatile and a nonvolatile oil, yielding 3% and 18% by weight, respectively, of starting material were formed when Cheshire *et al.* (1968) heated 1 g of acid-boiled humic acid in a sealed tube at 250 °C with 1 g of red phosphorus and 30 cm^3 of HI (density 1.74). When the oils were dehydrogenated with a palladium–carbon catalyst, or with sulphur, a number of fused aromatic structures were formed. This was thought to substantiate the zinc-dust distillation data, and indicated the presence of fused aromatic structures in the polymer. These promising techniques, initiated by Haworth and his group, are well worth further investigations.

Degradation by hydrogenation and by hydrogenolysis. Oils and in some cases cyclopentanols, phenols, and phenolcarboxylic acids have been reported from hydrogenation of humic acids under pressure (Ganz, 1944; Gottlieb and Hendricks, 1945; Kukharenko and Savel'ev, 1951 and 1952; Kukharenko and Kredenskaja, 1956; Murphy and Moore, 1960).

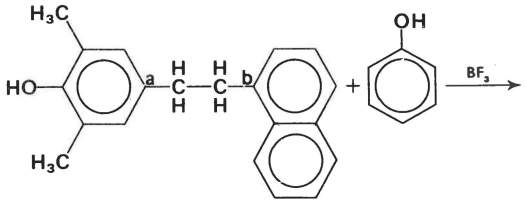
In carefully controlled work, which included investigations of possibilities for artefact formation, Felbeck (1965a; 1967) subjected an acid hydrolysed organic (muck) soil to hydrogenolysis at 350 °C and 41 Nm^{-2}. He isolated 50–60 % of the carbon in the starting material as hexane- and benzene-soluble materials. The products contained C11 to C35 n-hydrocarbons, and the C22 to C32 compounds were present in greatest abundance. Calculation of the carbon-preference index (CPI) for the data for the C12–C35 hydrocarbons gave an odd/even CPI value of 0.96 which contrasted with the values (3–8) for most biologically derived materials. His data would indicate that substantial fragments of the polymer cleaved at the weaker bonds, and he proposed that γ-pyrone structures in humic substances might account for the behaviour observed (Felbeck, 1965b).

Degradation with sodium and liquid ammonia. Maximov and Krasoskaya (1977) found that from 9 to 21 % of the substrates were degraded to water-soluble products when 6M HCl hydrolysed humic acids from a forest soil were reduced by metallic sodium in liquid ammonia at −33 °C. These amounts were increased to *ca.* 45 % when the reduction experiments were repeated five times on each substrate. However, only 10.5 % of the products were ether-soluble. By use of g.l.c. and g.c.–m.s. techniques, hydroxy- and dihydroxybenzenecarboxylic acids, hydroxybenzene polycarboxylic acids, and benzenecarboxylic acids were detected in amounts totalling 32.5 mg g^{-1} of humic acid. They postulated that such compounds could have been released from ether linkages involving the foregoing structures.

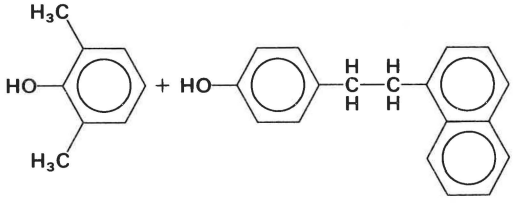
Degradation with phenol

Herédy and Neuworth (1962) introduced this technique to coal science when they showed that 60 % of a bituminous coal (80.4 %C) was depolymerized at 100 °C by phenol and boron trifluoride catalyst. Later Ouchi *et al.* (1965) found that a Yubari coal (84.6 %C) was almost completely depolymerized when refluxed for 27 hours at 185 °C (the boiling point of phenol) in the presence of *para*-toluenesulphonic acid (PTS) catalyst.

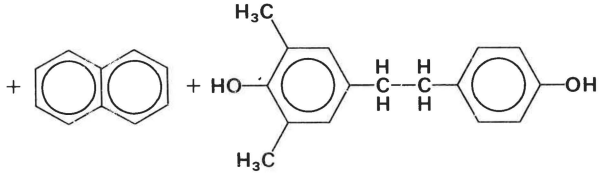
In a reaction with the model substance 1-(4-hydroxy-3,5-dimethylphenyl)-2-(1-naphthyl)ethane (**IL**) Henédy and Neuworth (1962) proposed that clearages took place as outlined in reaction scheme (3.21). The products expected would arise from attack on bonds a and b. Their results showed that bond a was particularly susceptible to cleavage, as determined by the recovery of 2,6-dimethylbenzenol (**L**), but the small amount of naphthalene released suggested



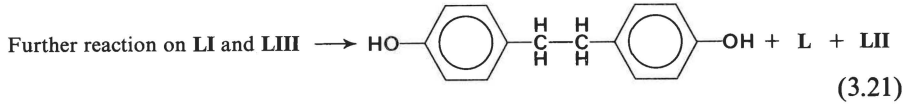
(IL)



(L) (LI)



(LII) (LIII)



(3.21)

that bond b was relatively resistant to cleavage. However, when the ethylene linkage in **IL** was replaced by a methylene ($—CH_2—$) group 73% and 41% of the theoretical yields of **L** and **LII** were obtained, respectively, showing that in this instance significant cleavage of bond b had taken place.

Jackson, Swift, Posner and Knox (1972) introduced the phenol–PTS method, for the degradation of soil humic acids. After refluxing for 24 hours excess phenol and PTS were removed by steam distillation, and the petroleum ether-soluble fraction of the methylated residue was separated by column chromatography, preparative g.l.c. and thin layer chromatography. The separates were identified by chemical and spectroscopic methods. Figure 3.8 presents a list of the compounds identified. Compounds **122**, **126**, **127**, **130**, and **131** were also identified (Ouchi and Brooks, 1967; Imuta and Ouchi, 1968, 1969) among the degradation products of coals.

From the few model studies which are available we can predict some polymer linkages which would cleave to give the structures in Figure 3.8. The xanthene

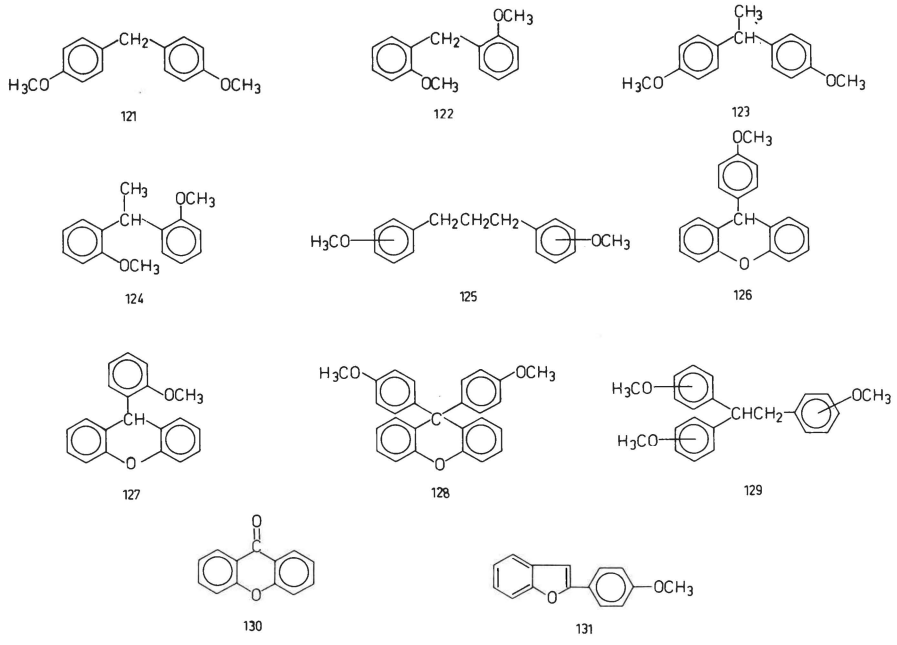
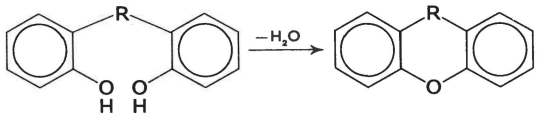


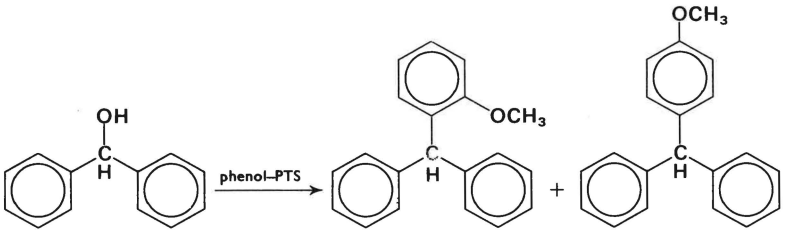
Figure 3.8 Products identified in the digests from phenol + *p*-toluenesulphonic acid–humic acid degradation reactions. (After Jackson *et al.*, 1972)

structures, 9-(4′-methoxyphenyl)xanthene (**126**), 9-(2′-methoxyphenyl)xanthene (**127**), 9,9-bis(4′-methoxyphenyl)xanthene (**128**) and xanthone (**130**) were formed by dehydration of appropriate 2-hydroxy benzene structures as indicated in reaction scheme (3.22), where R is a bridging (usually aliphatic) structure. Clearly the formation of xanthene and xanthone structures indicate that aromatic ether linkages are not broken. Also the 1-naphthylmethyl ether linkage remained intact in the model studies, and this suggests that aliphatic–aromatic ethers are not cleaved. Thus the bis(4-methoxyphenyl)methane (**121**), bis(2-methoxyphenyl)methane (**122**), 1,1-bis(4′-methoxyphenyl)ethane (**123**), 1,1-bis(2′-methoxyphenyl)ethane (**124**), and 1,3-bis(methoxyphenyl)propane (**125**) compounds could have formed from structures in which aromatic groups were linked by the appropriate aliphatic bridges.



(3.22)

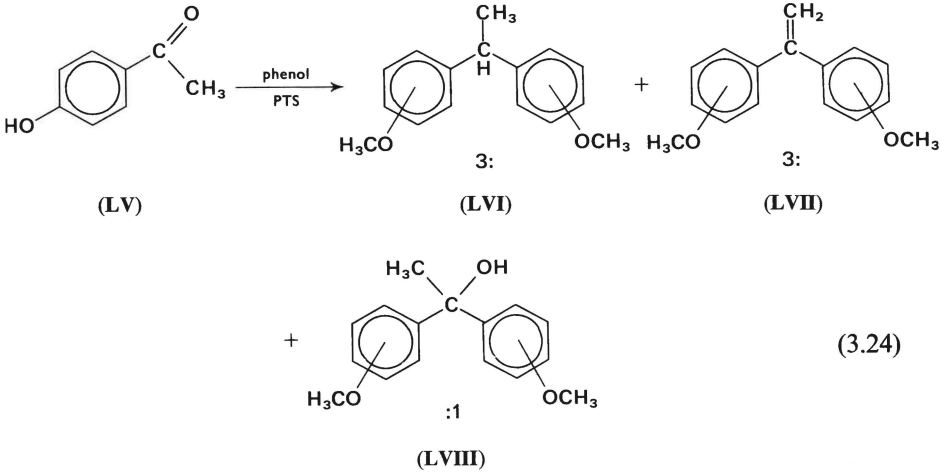
In a model study with biphenyl methanol, or benzhydrol (**LIV**), using the phenol–PTS system, Piper and Posner obtained, after methylation, the products



(LIV) 30% 40% (3.23)

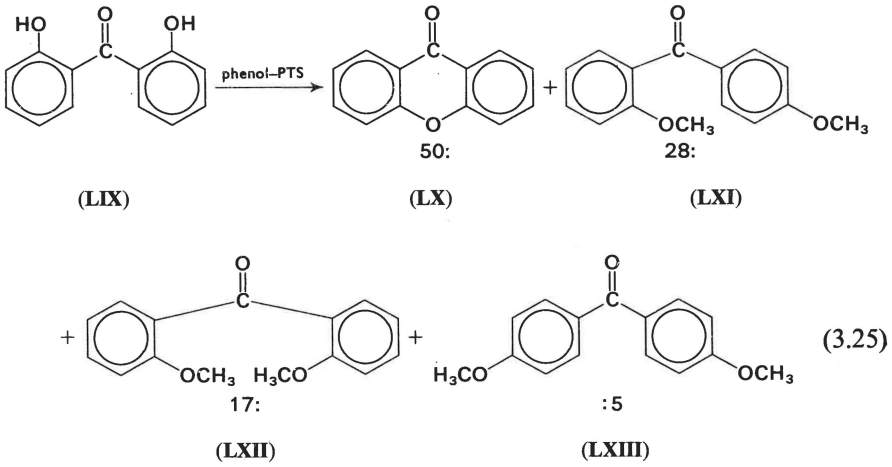
in the yields indicated in reaction scheme (3.23). There it will be noted that benzene substituents were not cleaved and that the —OH was displaced by the phenol. This provides an alternative explanation for the origins of compounds **121–125**, which could also be formed from hydroxyl groups on terminal carbon atoms. Similarly 9-(4′-methoxyphenyl)xanthene (**126**), 9-(2′-methoxyphenyl)xanthene (**127**) and 1,1,2-tris(methoxyphenyl)ethane (**129**) could have been derived from secondary alcohols. The cyclic ether structures were formed from *ortho*-hydroxybenzenes in the polymer, or more likely from *ortho*-phenol groups substituted during the reaction. Alternatively these structures could be formed by displacement of triaromatic substituents on the tetrahedral carbon.

In another model study 4-hydroxy acetophenone (**LV**) cleaved to products in the ratios indicated by the methylated structures in scheme (3.24) (Piper and Posner, 1975). This reaction suggests that a methyl ketone origin is possible for structures **123** and **124**, and indicates, from compound **LVII**, that where enols can form, the hydroxy group is replaced by phenol.



(3.24)

When 2,2′-dihydroxybenzophenone (**LIX**) was reacted with phenol in 1:10 ratio (Piper and Posner, 1975), four products were identified in the proportions



(LIX) (LX) (LXI)

(LXII) (LXIII) (3.25)

given in reaction scheme (3.25). This indicates that xanthone (**LX** and compound **130** in Figure 3.8) was derived from a diaromatic ketone structure in the humic acid molecule. Although some 9,9′bis(4-methoxyphenyl)xanthene (compound **128**) was formed when xanthone was reacted in a 1:110 ratio with phenol, this could not account for the amounts present in the humic acid digest. It would therefore appear more likely that **128** was formed from a triaryl tertiary alcohol structure, or less likely from a quaternary aryl group. In either case this would represent a potential branching point in the polymer structure. Other possible branching points could be indicated by structures **126**, **127**, and **129**, if it is assumed that hydroxyl was absent from the original (R) linkages.

No convincing explanation can be offered for the origins of **131**, but it could be formed from a lignin-type percursor.

The phenolic degradation technique differs from the others discussed because it helps to identify interaromatic linkages. As such it is complementary to other degradative techniques which are primarily used to identify monomer structures. Yield figures indicate that this reaction causes extensive degradation, giving approximately 50% ether-soluble components, and containing numerous, as yet unidentified products. There is obviously great scope for further research on this technique.

Degradation with sodium sulphide

Mixtures of sodium hydroxide and sodium sulphide have been used in the pulping industry since the end of the last century to delignify hardwoods and softwoods. These chemicals are the main ingredients of Kraft pulping liquor, and lesser amounts of sodium carbonate, sodium thiosulphate, and sodium polysulphide are also present. The potential value of sodium solutions for degradation of humic substances was examined by Swift (1968) and was further developed by Craggs (1972).

Hayes *et al.* (1972) isolated 60 % of the starting polymer mass as ether and ether–ethanol soluble products when H^+-humic acid was degraded in a 10 % sodium sulphide solution at 250 °C. Craggs *et al.* (1974) reported similar yields of ether-soluble components, and these were composed of materials in solution (45 %) and associated with the precipitate (15 %) formed when the digest was acidified. The average molecular weight values of the acid soluble and insoluble materials were 145 ± 15 and 245 ± 25, respectively, and these values indicated that the digestion procedure had caused extensive degradation of the polymer. More recently O'Callaghan (1977) has shown that yields of ether-soluble products are improved when cobalt molybdate (a dehydrodesulphurization catalyst) is added and the reaction is carried out in an atmosphere of hydrogen. It is thought that the reducing conditions inhibit oxidative coupling of phenols which give rise to artefact products.

Marton (1971) has summarized a number of model studies of reactions of sodium sulphide with compounds related to structures in lignins, and Craggs (1972), Burdon *et al.* (1974) and Craggs *et al.* (1974) have investigated some reaction mechanisms during the course of their humic acid degradation reactions.

$$S^{2-} + H_2O \overset{K_1}{\rightleftharpoons} HS^- + HO^-;\ HS^- + H_2O \overset{K_2}{\rightleftharpoons} H_2S + HO^- \qquad (3.26)$$

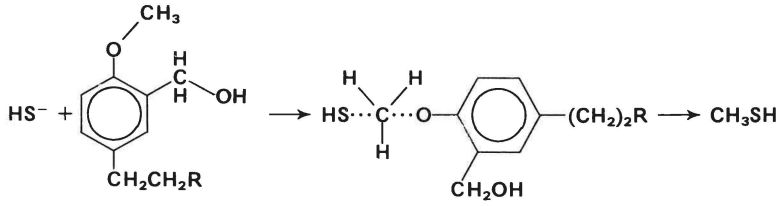
It is important to understand the solution properties of sodium sulphide before considering any of its mechanisms of degradation. The compound hydrolyses in water as shown in scheme (3.26) where the equilibrium constants in the two reactions, K_1 and K_2, are the first and second dissociation constants of hydrogen sulphide.

The values for pK_1 and pK_2 indicate that negligible amounts of S^{2-} are present in the alkaline medium (pH = 12.5) at high temperatures, and the HS^- and HO^- species are both present in similar concentrations. Nucleophilic strength increases in the order $HO^- < HS^- < CH_3S^-$, and this can be explained on the basis of the polarizability of the anions; the larger HS^- ion being more readily polarized than the smaller HO^- species. The ability to abstract a proton, or the basic strength, is in the reverse order.

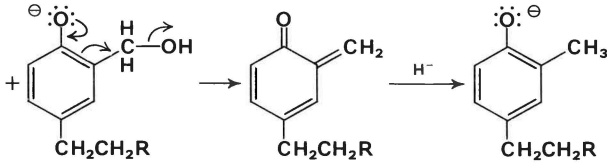
Important features of sodium sulphide degradation reactions include:

(i) demethylation of methylphenyl ethers (a well known reaction in lignin chemistry);
(ii) the formation of quinone methide structures;
(iii) hydride transfer processes (Weedon, 1963);
(iv) the partial inhibition of condensation reactions (Marton, 1971).

Reaction scheme (3.27) incorporates the first three of these features. Attack by HS^-, in an S_N2 mechanism leads to the formation of methyl mercaptan (CH_3SH) and the anion species (**LXV**). This anion can lose OH as indicated to form the *ortho* quinonemethide structure (**LXVI**) which takes up hydride to give **LXVII**.

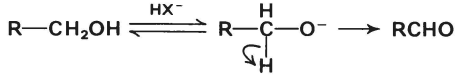


(LXIV)



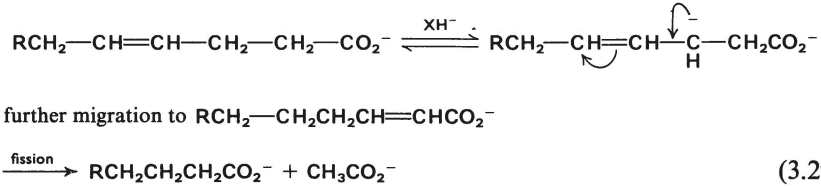
(LXV) (LXVI) (LXVII) (3.27)

A number of mechanisms can give rise to hydride formation; one of these is the Dumas–Stass reaction (3.28) where the base HX^- is HS^- or HO^-. The HS^- can also accept H^- to release H_2 and give the S^{2-} species. Methyl mercaptan is ionized to the mercaptide in the alkaline medium, and the highly nucleophilic mercaptide can attack another methyl phenylether group to form the phenate anion and dimethylsulphide (CH_3SCH_3).



(3.28)

Several aliphatic compounds were identified among the more volatile components in the lower molecular weight fraction of the sodium sulphide digest of humic acid. These included aliphatic mono- and dicarboxylic acids, and some keto acids (Craggs *et al.*, 1974). Many of the products which had longer retention times during g.l.c. separation were aromatic and had spectral properties indicative of phenylpropane-type structures. It was considered unlikely that these were artefacts since no aromatic compounds were formed when cellulose was degraded by the same technique. By taking into account the glucose equivalent of the carbohydrate (7 %) associated with the humic acid material it was concluded that the short chain aliphatic acids identified were primarily derived from the humic polymer and not from associated carbohydrate.

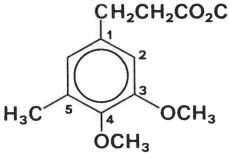


(3.29)

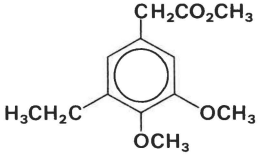
Ethanoic acid was present in greatest abundance in the digest. One plausible explanation would suggest that this and other acids might have formed by a Varrentrapp reaction as outlined in scheme (3.29).

This scheme illustrates the reversible migration of the double bond by a protropic mechanism, and the mobility of the double bond increases as it approaches the carboxylate group. When the double bond reaches the α, β position fission takes place following attack by base to give the β-hydroxy or thiol acid.

Compounds **LXVIII** and **LXIX** are representative of a number of the methylated aromatic species isolated from the digest and identified or tentatively identified. On the basis of the demethylation illustrated in scheme 3.27 we can assume that the methoxyl substituents were present in the digest as phenolic hydroxyls prior to methylation. Thus quinone methide structures would be formed where appropriate substituents were located *ortho* and *para* to the hydroxyl groups. Reference again to reaction scheme (3.27) will show how the methyl substituent on the aromatic C_5 should have formed from $—CH_2OH$ (which could in turn have formed from the carbaldehyde structure). More significantly, perhaps, it could also have formed from elimination of an α-ether substituent, and the reduction of the resulting quinone methide.



(LXVIII)



(LXIX)

The ethyl substituent on aromatic C_5 of **LXIX** could have formed from the secondary alcohol, α-ethers, or methyl ketone structures. In the case of the ketones, hydride reduction would yield the secondary alcohol.

Quinonemethide structures could also be formed in cases where alcoholic hydroxyl, keto, and ether substituents were present on the α-carbon attached to aromatic C_1 in **LXVIII** and **LXIX**. However, it is likely that the esters were formed after cleavage of aliphatic side-chains to yield the carboxylic acid structures. Again the Varrentrapp reaction, or reaction scheme (3.18) by Shemyakin and Shchukina (1956), could be invoked to explain the formation of these carboxyl groups, but further studies with model compounds will be required before definite conclusions can be reached with regard to their possible origins.

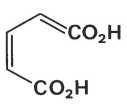
Clearly the sodium sulphide degradation technique can provide very useful information with regard to the primary structures of humic substances. At its present stage of development the technique strongly indicates that considerable amounts of aliphatic substituents are attached to the aromatic structures in these materials. Quantitative isolation and identification of digest products, coupled with extensive studies of reactions of model compounds are now

required in order to be able to appreciate its full potential as a degradative procedure.

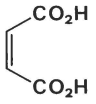
Additional degradation procedures

Peracetic acid oxidation, anodic oxidation, and pyrolysis procedures provide additional useful techniques for the degradation of humic substances. Although the first two procedures have extensive uses in organic chemistry they have only had limited applications to investigations of humic structures. There is, however, a growing interest in pyrolysis, and this developing technique appears to be especially useful for making comparisons between the compositions of humic materials of different origins.

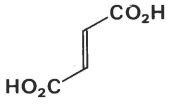
Peracetic acid (CH_3CO_3H), when protonated dissociates to the reactive OH^+, and this species, as well as the protonated parent peracid, takes part in oxidation reactions. Well known reactions of peracids include the formation of oxiranes from alkenes, the conversion of ketones to esters and lactones, and the oxidation of aliphatic aldehydes to carboxylic acids.



(LXX)



(LXXI)



(LXXII)

Of special interest here is the fact that phenols and phenol ethers readily undergo electrophilic substitution when treated with peracetic acid. Substitution is *ortho* and *para*, and the dihydroxy benzenes form quinone structures which oxidize further to dicarboxylic aliphatic acids. For instance 2-hydroxybenzenol yields muconic acid (**LXX**) and 4-hydroxybenzenol gives the *cis* and *trans* butenedioic, or maleic (**LXXI**) and fumaric (**LXXII**) acids. Waters (1964), Behrman and Edwards (1967), and Lewis (1969) have reviewed the mechanisms of peracid oxidation reactions.

Chang and Allan (1971, pp. 458–459) have described the uses of peracetic acid for the degradation of lignins and wood, and they have presented some relevant mechanisms for reactions with model substances. Meneghel *et al.* (1972) oxidized a peat humic acid with peracetic acid for eight days at 40 °C and identified five amino acids and six sugars in the digest. Schnitzer and Skinner (1974a, 1974b) were more successful when they heated chernozem humic and fulvic acids for four hours in 10 % peracetic acid at 80 °C. Among the compounds identified were numbers **1**, **2**, **3**, **8**, **9**, **10**, **11**, **13**, **14**, **16**, **17**, **18**, **19**, **20**, **21**, **23**, **24**, **25**, **26**, and **27** in Figure 3.4, and numbers **53** and **62** in Figure 3.5. Other compounds included propanedioic acid, 2-methoxy-1,5-benzenedicarboxylic acid, 3,4-dimethoxy-1,5-benzenedicarboxylic acid, 5-methoxy-1,2,3-benzenetricarboxylic acid, nC_{12}–nC_{20} fatty acids, methyl-3,4-furandicarboxylic acid, and branched chain butanoic acids.

Total yield of products and the types of structures identified were very similar to the results from degradations with permanganate. Again the results would suggest that aliphatic substituents on the aromatic structures were oxidized. It will be necessary to carry out model studies on possible precursors of the identified products before predictions can be made with regard to their parent structures in the humic polymers.

Ross *et al.* (1975) have extensively reviewed electrochemical oxidation reactions of organic compounds and illustrated numerous mechanisms of anodic oxidations. Strangely, despite the promising application by Belcher (1948) of anodic oxidation to coal fractions, the technique has not been adequately tested for the degradation of soil humic substances. Yields of up to 35 % ether-soluble products were obtained by Belcher when he oxidized vitrain coal materials at the copper anode. Some benzene polycarboxylic acids, similar to those produced by permanganate oxidation, were isolated and identified.

Modern separation and instrumental analysis procedures would allow identification of a high proportion of the products released during controlled anodic oxidation reactions. Thus, by step-wise increases of the electrode potential (and analysis of products released during each increase) it might be possible to observe differences in the strengths of bonding in the polymer of the products released at different anode potentials. The resistant residues could possibly be further degraded by chemical procedures.

Volatile degradation products are released when polymers are rapidly pyrolysed in an inert atmosphere. These products may be separated by g.l.c. and analysed by m.s. or g.c.–m.s. instrumentation (Meuzelaar *et al.*, 1974) and the traces obtained can be used to 'fingerprint' the polymers.

Pyrolysis technology has been greatly advanced by the development of Curie point pyrolysis procedures (Bühler and Simon, 1970). This allows samples in the microgram range to be heated at rates in excess of 1 °C $msec^{-1}$, and the possibilities of secondary reactions are minimized by the rapid removal of the degradation products from the reaction zone.

For more meaningful interpretations of results it is important to be able to predict the types of parent polymer structures which give rise to the degradation products. Thus considerable exploratory work is still required in order to evaluate fully data from the pyrolysis of humic substances. At this time interpretations are based on information related to non-humic systems, such as proteins, fats and waxes, carbohydrates, and lignins. According to Simmonds *et al.* (1969) the pyrolysis of proteins, peptides, and amino acids give nitriles, fats and waxes produce unbranched alkanes and alkenes, and carbohydrates give aliphatic aldehydes, ketones, and furan derivatives containing $—CH_2OH$, —CHO, and CH_3 substituents. Because the organic materials in ancient sediments and meteorites pyrolyse to predominantly hydrocarbon-type products compared to the more heteroatomic fragments which characterize present-day biologically derived materials, pyrolysis gas chromatography was

chosen to examine Martian soils for evidence of materials from life systems during the Viking mission in 1975.

There are already a number of applications of pyrolysis techniques for studies of soil organic matter, and of soil and model humic substances (Nagar, 1963; Wershaw and Bohner, 1969; Kimber and Searle, 1970; Gomez Aranda *et al.* 1972; Murzakov, 1972; Bracewell and Robertson, 1973 and 1976; Martin, 1975; Nagar *et al.* 1975; Meuzelaar *et al.*, 1977; Martin *et al.*, 1977; Haider *et al.* 1977a). Bracewell and Robertson (1976) have given a good example of the uses of pyrolysis g.l.c. for 'fingerprinting' humus materials from different soils and in different horizons in the same soil. By use of a Curie-point pyrolyser linked to g.l.c. they were able to observe chromatogram differences between mull and mor humus of whole-soil samples. When the effluent from the g.l.c. column was analysed by mass spectrometry the identified products included furfural, pyrrole, 5-methyl-2-furfuraldehyde, benzenol, 2-methylbenzenol, 2-methoxybenzenol, and 4-methyl-2-methoxybenzenol. A lignin product gave significant yields of 2-methylbenzenol and 2-methoxybenzenol.

Mor humus produced considerable amounts of furfural derivatives, possibly indicating a carbohydrate source. By contrast pyrrole was the main component of mull humus and this could suggest an origin in proteins, or the amino acids proline and hydroxyprolines, porphyrin structures or soil microorganisms (Simmonds, 1970). The patterns for the relatively undecomposed surface organic matter suggested the presence of lignin, but the characteristic benzenol derivatives were not present to a significant extent in the humus in the mineral soil horizons.

By use of Curie-point pyrolysis in direct combination with a fast-scanning quadrupole mass spectrometer Meuzelaar *et al.* (1977) and Haider *et al.* (1977a) were able to show definite similarities and differences between humic acids from soils, peats, model phenolic polymers, fungal 'humic acids' and lignins. Again their results emphasize the value of pyrolysis techniques for 'fingerprinting' humic substances.

Further development of pyrolysis procedures could allow some interpretations to be made relative to the structures of humic substances. This will, of course, require extensive model studies with known polymers and chemicals pyrolysed under the same reaction conditions as used for the humic materials. Such an approach is encouraged by the work of Martin (1975) who identified 18 organic compounds among the low-boiling components in the chromatogram from pyrolysis g.l.c. of humic and fulvic acids. Furane derivatives were not detected when the samples were pre-hydrolysed, which indicates that carbohydrates were removed by the hydrolysis process. The 12 aliphatic alkanes and alkenes, with up to 10 carbon atoms, suggested aliphatic or/and alicyclic sources. Benzene and methylbenzene were present in all pyrolysates, but their amounts decreased after hydrolysis. On the other hand benzenol increased on hydrolysis, and this could have been derived from lignin and from humic structures.

General conclusions from degradation studies

Considerations of digest yields of ether- and ethylacetate-soluble products from degradations of humic substances by procedures employed until the present time emphasize the need for a continuing search for more efficient degradative reagents and conditions. It is unlikely that complete degradation to molecules which can be related to structures in the polymer will be achieved by use of a single reagent, and it is probable that a sequence of reactions will be required in order to obtain maximum yields of component products.

Hydrolysis in acid and base can be regarded as a preliminary treatment (Jakab *et al.*, 1962) which cleaves labile structures such as the glycosidic linkages of oligo- and polysaccharides (Section 3.2.6), ester groups from condensations between phenolic hydroxyls (as exemplified in structure **XXX**) or alcohols and carboxylic acids, and the peptide linkages bonding amino acid residues in proteins and polypeptides. Components held in these ways can be regarded only as peripheral to the structures of humic substances. Carbohydrates, for instance, are seldom present in excess of 10 per cent of the total mass of humic acids, and could be bound to them by physical adsorption forces, or more likely through phenolic glycoside linkages. Peptides or proteinaceous materials, which are present in lesser abundances than the carbohydrates, could also be physically adsorbed, but they are more likely to be bonded to quinone-type structures through terminal or free amino groups as indicated by scheme (3.1), or to carbonyl groups through Schiff-base type linkages as indicated by reaction schemes (3.5) and (3.6).

Cleavage of the 'core' or fundamental structures of humic substances is energetically demanding. This would not be true, as Maximov *et al.* (1977) have also pointed out, if the acid compounds shown in Figures 3.4 and 3.5 (or, for that matter, similar compounds among any of the digest products listed) were *per se*, the 'building blocks' of the 'core' structures. Although such acids could be held together when undissociated through hydrogen bonding they would be dispersed when solubilized under neutral or alkaline conditions and would not exhibit polymer properties. Because of the relatively high energy requirements for the degradation of humic polymers it is probable that carbon to carbon, carbon to oxygen (e.g. ether), and possibly some carbon to nitrogen bonds are important in linking together the monomer units or primary structures of humic materials.

Techniques which employ phenol plus *para*-toluenesulphonic acid and sodium sulphide at moderately elevated temperatures cleave humic acid polymers to ether-soluble components amounting to 50–60 per cent of the masses of the starting materials. However, the major proportions of the products released by these procedures have not yet been identified. Polymer degradations have not been as extensive from controlled oxidations with permanganate, alkaline cupric oxide, and peracetic acid, but the use of modern separation and spectroscopic techniques (as employed by Schnitzer and his colleagues) has helped to identify most of the ethylacetate and ether-soluble digest digest products which

can amount to 20–30 per cent of the polymer masses. But even when allowances are made for the fact that product yields in organic reactions are generally depressed, it is obvious that the bulk of the polymer masses are not degraded to monomer components by the foregoing oxidative processes. Considerations of yields and of identities of degradation products do not provide sufficient information to allow predictions of meaningful structures for the polymers. Nevertheless, by taking into account the types of compounds identified from a variety of digest reactions in several laboratories, some predictions can be made about some of the humic polymer component molecules, and about the types of linkages which hold these together.

There is unquestionable evidence for the presence of substituted single aromatic structures in humic polymers. These are almost invariably di- or polysubstituted, because only traces of monosubstituted benzene derivatives, such as benzoic acid, have occasionally been detected in digests. Proof that fused aromatic compounds contribute to the structures is less convincing. The available evidence shows that such compounds, which are present only in small abundances in the digests, could have been formed as artefacts during the degradation (e.g. zinc-dust distillation and fusion) reactions. Some of the aromatic di- and polycarboxylic acids (Figure 3.4) from the permanganate degradations might also suggest fused aromatic parent structures, but it is noticeable that similar acids were formed or released in reactions (such as with alkaline cupric oxide, Figure 3.5) in which the fused structures would not be degraded to benzenecarboxylic acid derivatives. Thus we favour the interpretation that at least some of the substituent carboxyl groups were derived by oxidations of aliphatic side chains.

Functional group analysis data have shown that humic polymer structures contain carboxyl, phenolic and alcoholic hydroxyl, carbonyl and methoxyl groups. Some of the carboxyl groups are unquestionably present as substituents on the aromatic structures in the polymer, as can be inferred from compounds identified in digests of sodium amalgam reduction reactions (Figure 3.6). It is unlikely, however, that the aromatic nuclei in the polymer were polysubstituted with carboxyl groups, as seen in Figures 3.4 and 3.5, and it is more plausible to suggest that these were generated from aliphatic side-chains. There is evidence for the presence of such side-chain structures in the cases of some groups which resisted oxidation by permanganate and alkaline cupric oxide. However, the products from sodium sulphide degradation reactions give the strongest indications for the presence of more than one aliphatic side-chain substituent on aromatic nuclei.

There are striking differences between the types of products released by oxidative and reductive processes, and these differences must be related to the linkages which are cleaved in the parent polymer structures by the different reagents. Degradations with sodium amalgam released considerable amounts of di- and trihydroxybenzene derivatives, and these sometimes contained carboxyl (but never more than one group per aromatic structure), methoxyl,

carbonyl, methyl and longer aliphatic chain substituents. It is possible that some (but perhaps not all) of the polyphenol derivatives identified had been decarboxylated during the degradation reactions. But clearly, with the exception of structure **85**, none of the products from the amalgam reductions in Figure 3.6 could be oxidized to aromatic structures containing more than one carboxyl group, and this contrasts with most of the structures isolated from oxidation reactions (listed in Figures 3.4 and 3.5). Therefore the available evidence indicates that different polymer structural units are released by the oxidation and reduction reactions, further indicating that different linkages are attacked although, of course, some overlaps could occur.

Diaryl ethers have been shown to be cleaved in sodium amalgam degradations, and all of the compounds listed in Figure 3.6 (where allowance is made for methylated products) could have been released from such ether linkages in the humic polymers. Additional model studies will be required in order to postulate other polymer origins for the various structures, but it can be stated with confidence that the carbonyl and carboxyl structures shown reflect active functional groups in the polymer, and do not represent artefacts formed in the digest mixtures. Other interesting amalgam digest products include phenylpropane units containing methoxyl and hydroxyl substituents on the aromatic nuclei, as such structures might originate from lignin-type materials.

Examination of the aromatic compounds obtained by the oxidative degradation processes shows that many of these are di- and polycarboxylic, and that several also contain methoxyl and/or hydroxyl substituents; it is impossible to estimate the free hydroxyl group contents in these instances because the digest products were methylated prior to analysis. Aromatic permanganate degradation products were especially rich in carboxyls, and it is plausible to assume that several of these were formed by the oxidation of aliphatic side chains. The same digests yielded considerable amounts of di- to tetracarboxylic acids which could have been formed from olefinic structures appropriately separated by $-CH_2$ groups, unsaturated branch chains, and/or aldehyde or primary alcohol substituents. Such structures could be bonded to the aromatic components through various ester and ether linkages, or they could of course be held as hydrocarbon substituents on the aromatic nuclei.

Oxidations with alkaline nitrobenzene and alkaline cupric oxide provide further evidence that some of the digest products might have been released from lignin-type units in the polymer structures. This is especially obvious in the cases of the compounds (see Figure 3.5) which contain carbonyl groups as well as methoxy substituents in the aromatic 3,4- or 3,4,5-positions. Like permanganate, alkaline cupric oxide digestion yielded di- to tetracarboxylic acids, but the latter reagent also produced significant numbers and yields of aliphatic hydrocarbons and monocarboxylic acids. Mechanisms, such as those in reaction schemes (3.18) and (3.29, the Varrentrapp reaction), show how both types of products could have been obtained in alkaline media from olefinic structures.

We cannot confidently predict whether or not the long-chain aliphatic hydrocarbons and acids were derived from polymer structures, or whether they should be regarded as humification products. They could merely represent unaltered products of microbial metabolism which were physically adsorbed to the humic structures and freed during the degradation processes. Alternatively (and very likely) the acids were released from ester structures, as has been discussed, by the hydrolytic action of the aqueous base media. It would appear unlikely that such long chain aliphatic structures are substituted into the aromatic structures, although we have no evidence that they were not.

Degradations with phenol plus *para*-toluenesulphonic acid suggest that aromatic groups in humic polymers could be linked by hydrocarbon bridges. The structures shown in Figure 3.8 represent only a small proportion of those released in the digests, but it can be inferred from the limited data available that straight-chain and branched hydrocarbon structures could be involved in such linking processes. Alcoholic hydroxyls could also have given rise to some of the structures identified.

On the basis of the information which is available at this time we conclude that most can be learned, in so far as humic structures are concerned, from degradations with alkaline cupric oxide, sodium amalgam, sodium sulphide, and phenol. It may well be worthwhile to investigate the effects of the different reagents applied in sequence. Such investigations would demand rigorous anaerobic conditions where alkaline media are used because of the possibilities of secondary polymerization reactions which are accelerated by the presence of oxygen.

3.4.4 Chemical and Biological Synthesis of Humic Substances

Most of the work directed towards understanding the structures of humic materials has concentrated on degrading the polymers and identifying the compounds released into the digests. It is obvious from the structures listed in Figures 3.4–3.8 that very many products, real or artefacts, can be detected by these procedures. An alternative approach would be to chemically, enzymatically, or biologically synthesize humic-type substances from known starting materials, and to compare their compositions and physical and chemical properties with those of natural humic polymers.

Chemical synthesis of 'humic' polymers

Maillard (1912, 1916, 1917) observed that brown polymers formed during the reaction of glucose with glycine (2-aminopropanoic acid) displayed some properties similar to those of soil humic acids. This reaction, classically known as the 'Maillard' or 'browning' reaction, is well recognized in food processing industries, and has been reviewed by Hodge (1953) and Ellis (1959).

Several reactions are known in which amino acids and amines react with

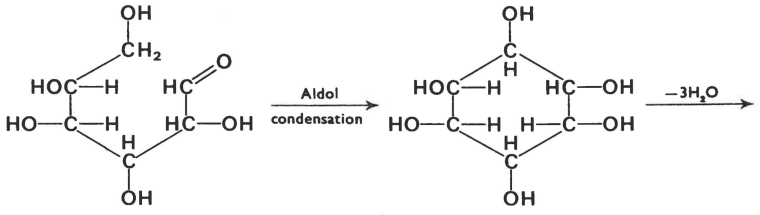
reducing sugars and aldehydes to give melanoidins or 'humic-like' substances. Among the more interesting products are polymers formed by the reaction of propanedione (methylglyoxal) and glycine which Enders and his colleagues (Enders and Fries, 1936; Enders, 1943a, Enders *et al.*, 1948) have shown to possess properties comparable with soil humic acids. Enders (1943b) also postulated that methylglyoxal could be released from soil microorganisms in unfavourable growth conditions to form humic substances, and later showed (Enders and Sigurdsson, 1948) that the compound was present in ten of the sixteen soils tested.

Schuffelen and Bolt (1950) found that the C:N ratio of the products from the reaction of methylglyoxal and glycine varied with the relative concentrations of reactants used, and they isolated a polymer which compared favourably with a Dalgrund peat on the basis of this ratio, titration, and CEC data. Later Hayes (1960) showed that the polymer formed in an atmosphere of oxygen from equimolar proportions of the same reactants had C:N ratios, differential thermal analysis properties, paper electrophoresis mobilities, and infrared spectra similar to neutral pyrophosphate extracted humic acids from an organic soil. These comparabilities do not, of course, necessarily imply similarities in compositions, and more significant tests would involve investigations of the compounds released when the polymers are degraded by chemical or physical means.

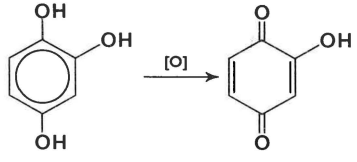
Glucosamine (see Section 3.6), a component of chitin, has been shown by Drozdova (1959) and Young *et al.* (1977) to form humus-like products when heated in solution, but it would appear that browning was not as rapid and the polymer products were not as high in molecular weights as those from the glucose– and methylglyoxal–amino acid systems.

Flaig *et al.* (1975) have reviewed the extensive literature which deals with the formation of dark coloured 'humus-like' substances from phenolic precursors. Phenols are regarded as important components because: (a) plant residues, such as lignins, which contain them are relatively resistant to degradation; (b) phenols or quinones are synthesized by microorganisms; and (c) phenolic lignin degradation products can be isolated from the soil.

Eller (1925) and his coworkers were among the first to observe some similarities between soil humic acids and the polymeric products formed by alkaline oxidation of phenols. The polyoxybenzene theory, reviewed by Flaig (1950), assumes that hexose sugars might aromatize to hydroxybenzoquinone as outlined in reaction scheme (3.30), and this highly reactive quinone structure (**LXXIII**) could readily polymerize under alkaline conditions by a number of different mechanisms. One of these might involve formation of ether linkages between the quinone structures followed by rearomatization as indicated in scheme (3.31). A number of studies, including those by Flaig and his colleagues (e.g. Flaig, 1960, 1966; Flaig and Schulze, 1952; Flaig *et al.*, 1955; Flaig and Salfield, 1958, 1960a, 1960b) have investigated the alkaline polymerization of hydroxy-*p*-benzoquinone and of hydroquinone and compared the products

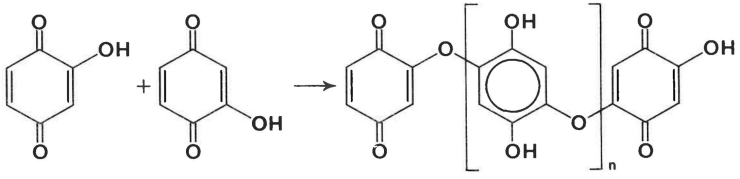


Hexose



(3.30)

(LXXIII)



(3.31)

with natural humic acids. Best yields of polymer are obtained in the pH 8–10 range. Formation of carboxyl group and decarboxylation can result from oxidation of the quinone structures. Incorporation of nitrogen into the polymers can also take place by a variety of mechanisms, one of which is outlined in reaction scheme (3.1).

It is, of course, highly unlikely that naturally occurring humic polymers would be wholly composed of chemically synthesized polyphenol or polyquinone structures. However, without doubt chemical synthesis could contribute to humus formation where a variety of phenols are present in the appropriate chemical environment in the soil. In this way chemical processes might supplement extracellular enzymatically catalysed reactions.

Enzymatic synthesis of 'humic' polymers

Martin and Haider (1977) have drawn attention to species of *Basidiomycetes*, *Ascomycetes*, *Fungi imperfecti* and some bacteria which can degrade lignin. They suggested that phenols released from lignins, microbially synthesized phenols, partially degraded lignins, and other reactive compounds could undergo autoxidation, or be enzymatically polymerized to humic molecules either extracellularly or within microbial cells. Other possibilities for humus

formation would link sugars and amino acids to reactive molecules by enzymatic or autoxidative processes.

Phenoloxidase enzymes are widespread and at least partially purified preparations can be isolated from the soil environment (Mayaudon and Sarkar, 1974). Mushroom phenolase, when added to mixtures of di- and trihydroxybenzenes, hydroxy- and methoxybenzenecarboxylic and -propenoic acids produce brown polymeric 'humic-type' structures (e.g. Martin and Haider, 1976, 1977; Verma and Martin, 1976; Haider *et al.* 1977b; Zunino and Martin, 1977). There is good reason to believe that such phenolic structures, released by degradation or biosynthesized, would be polymerized to humic materials by the phenoloxidase enzymes present, as well as by chemical reactions in the soil.

Microbial synthesis of 'humic' polymers

Numerous investigators have reported the formation of 'humic-like' substances by the activities of several fungal species, by bacteria, actinomycetes, and even by yeasts. Here we will concentrate on the products isolated by Martin and his associates from fungi grown on different media.

In 1967 Martin *et al.* cultured the fungus *Epicoccum nigrum* on a glucose–asparagine medium containing yeast extract and inorganic salts. After incubation for five weeks they isolated, at pH 2.0, mycelium-free 'humic acid' substances. Some of these were similar to a leonardite humic acid in elemental composition, total acidity, CEC, carboxyl and phenolic hydroxyl contents, and in molecular weight distributions, and the values reported were in the ranges quoted for soil humic acids. In soil incubation studies some of the synthetic polymers decomposed as little as 4 % in eight weeks (compared with 1–7 % in the same time for the leonardite preparations). The biosynthesized substances increased soil aggregation.

Subsequently Bondietti *et al.* (1971) showed that the addition of clays to similar media inoculated with the fungi *Hendersonula toruloidea* and *Aspergillus sydowi* accelerated growth, glucose utilization, and polymer synthesis. Montmorillonite was a more effective catalyst than kaolinite or illite. The catalytic effects of the clays were thought to result from (among other things) the concentration of enzymes and of substrates on the clay surfaces and the removal (also by adsorption) of metabolic wastes from the media.

Biosynthesis of fungal 'humic acid' precursors. It has been possible to follow the synthesis and degradation of phenols by various fungi by isolating at 4–7 day intervals the ether soluble products from fungal culture media. Figure 3.9, from Haider and Martin (1967), illustrates a plausible scheme for the genesis of 15 of the 20 or so phenols which were identified by two-dimensional thin layer chromatography from an *Epicoccum nigrum* culture. Compounds **132**, 2-methyl-3,5-dihydroxybenzenecarboxylic acid (cresorsellinic acid) and **133**,

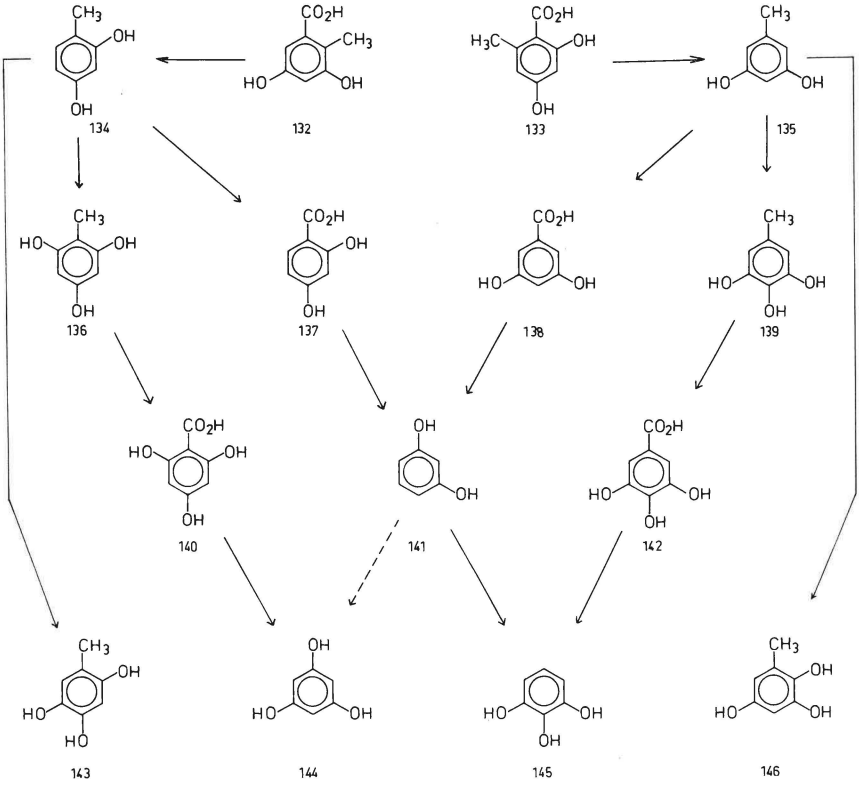


Figure 3.9 A scheme for the biosynthesis and transformation of phenols by *Epicoccum nigrum*. Reproduced from *Soil Science Society of America Proceedings*, Vol. 31, pp. 657–662 (1967), by permission of the Soil Science Society of America

2,4-dihydroxy-6-methylbenzenecarboxylic acid (orsellinic acid) and 1,4-dimethyl-2,6-dihydroxybenzene were first formed from non-aromatic precursors. Structures **132** and **133** can be biosynthesized either through the schikemic acid or acetate malonate pathways (Bentley and Campbell, 1968). These were quickly followed by **134** (2,4-dihydroxytoluene), **135** (3,5-dihydroxytoluene, or orcinol) and 1,4-dimethyl-2,6-dihydroxytoluene. Most of the remaining phenols in Figure 3.9 were formed during the later stages of incubation. By following the arrows it will be seen that some of the products identified could have been derived from precursors by oxidation of methyl substituents, by hydroxylation of the aromatic nuclei, and by decarboxylation of carboxylic acids. The unusual product 1,4-dimethyl-2,6-dihydroxybenzene might have been derived from non-aromatic precursors or from the introduction of a methyl group into the aromatic nucleus of orcinol (**135**).

Compound **133**, but not necessarily **132**, was found in the media in the early stages of incubation of other fungi. For instance the first products formed in a similar medium by *Stachybotrys atra* and *S. charatum* were **133**, *p*-hydroxy-

cinnamic acid (4-hydroxyphenyl 3-prop-2-enoic acid), and 2-hydroxy-6-methylbenzenecarboxylic acid (Martin and Haider, 1969). In the case of *Hendersonula toruloidea* (Martin *et al.*, 1972) **133** and 2-hydroxy-6-methylbenzenecarboxylic acid were formed at pH 3.5–4.5 from non-aromatic precursors, followed by **136**, **138** (3,5-dihydroxybenzenecarboxylic acid), **142** (3,4,5-trihydroxybenzenecarboxylic acid or gallic acid), and later by **141** (3-hydroxybenenol or resorcinol). The substitution of additional hydroxyl groups into the various aromatic

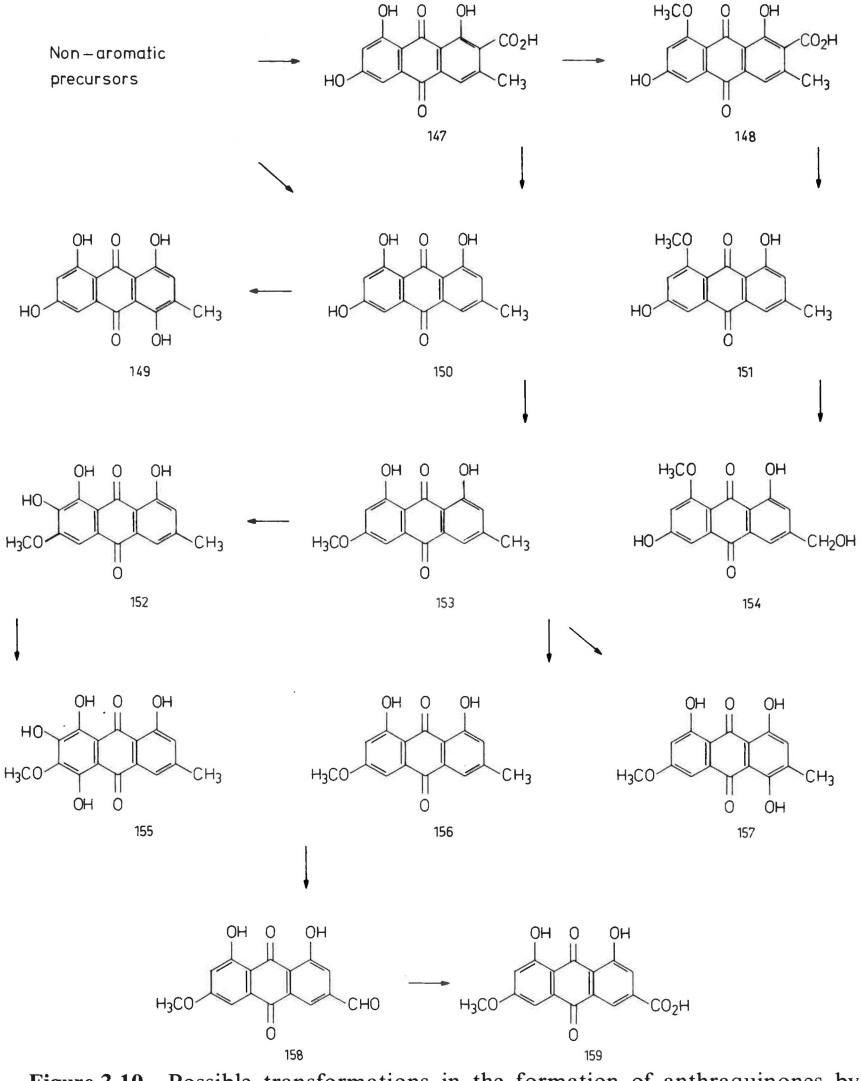


Figure 3.10 Possible transformations in the formation of anthraquinones by *Eurotium echinulatum*. Reproduced from *Soil Science Society of America Proceedings*, Vol. 39, pp. 649–653 (1975), by permission of the Soil Science Society of America

nuclei can result from strong mixed function oxidase enzyme systems (Haider and Martin, 1967).

Anthraquinones are synthesized by some fungi and Saiz-Jimenez *et al.* (1975) have identified the compounds shown in Figure 3.10, as well as several phenols in the ether extracts of cultures of *Eurotium echinulatum* grown on glucose–asparagine and glucose–nitrate media. Again the arrows lead from the possible precursors of the various anthraquinone derivatives identified. Compounds **147** (endocrocin) and **150** (emodin) can be formed from non-aromatic precursors by the acetate–malonate pathway (in Packter, 1973) and these can give rise to dermolutein (**148**), catenarin (**149**), questin (**151**), dermoglaucin (**152**), physcion (**153**), questionol (**154**), dermocybin (**155**), fallacionol (**156**), erythroglaucin (**157**), fallacinal (**158**) and parietinic acid (**159**). The first phenols formed, *p*-hydroxycinnamic acid and 4-hydroxybenzenecarboxylic acid were also synthesized from non-aromatic precursors by the shikimic acid pathway, and they were transformed by the appropriate oxidation, decarboxylation, and hydroxylation reactions into a range of phenolic compounds.

Resistance of precursors to microbial decompositions. By specific carbon-14 labelling of aromatic ring carbons, carboxyl groups, and aliphatic carbon side chains, Haider and Martin (1975), Martin and Haider (1976, 1977) and Haider *et al.* (1977b) showed that carboxyl groups in hydroxybenzenecarboxylic acids were readily decarboxylated in soil, that phenolic compounds with unsaturated aliphatic side-chains (models for constituents of lignin), such as 2(3-methoxy-4-hydroxyphenyl)propenoic acid (ferulic acid), 2(4-hydroxyphenyl)propenoic acid (cinnamic acid), 2(3,4-dihydroxyphenyl)propenic acid (caffeic acid), 3(3-methoxy-4-hydroxyphenyl)propen-1-ol (coniferyl alcohol), and 3(4-hydroxyphenyl)propen-1-ol (coumaryl alcohol) were more resistant to microbial decomposition in soil than glucose. The alcohols were significantly more resistant than the acids, and the aromatic nuclei had a relatively high degree of resistance and all could be substantially protected from biodegradation by incorporation into humic-type polymers. In the absence of polymerization, or of protection by polymeric materials, the various structures would degrade to ethanoic and butanedicarboxylic acids, and eventually to carbon dioxide and water. Thus it is probable that the primary structures are rapidly incorporated after synthesis into humic polymers in the soil.

Biosynthesis of fungal 'humic acid' polymers. Very little if any phenoloxidase activity and polymer formation takes place at the pH values of 3.5–4.5 characteristic of fungal culture media shortly after inoculation. Later, when media pH values rise to 5–6 pronounced phenolase activity takes place in the cells, and browning, indicative of polymerization takes place. Phenols and anthraquinones cannot be extracted from the medium during rapid polymer synthesis.

Raising the pH (to a limit of *ca.* 8.0) significantly increases the rate of polymerization, and also of autoxidation. Compounds such as 3,4,5-trihydroxytoluene (**139**), 3,4,5-trihydroxybenzenecarboxylic acid (**142**), 2,4,5-trihydroxy-

toluene (**143**), and 2,3,5-trihydroxytoluene (**146**) are capable of autoxidizing under alkaline conditions, and yield *ortho-* (and in the case of **146**, *para-*) quinone structures. Mention has already been made of chemical polymerization of quinone structures, and in particular of hydroxy quinones in alkaline media. Martin and his associates have drawn attention also to the possibilities of nucleophilic coupling of the quinones to give biphenyl and biphenyl ether structures.

General conclusions from humic synthesis studies

The information provided in this subsection has summarized how phenolic compounds in particular can be chemically or enzymatically polymerized to 'humic-type' structures. These phenols could arise from degradation of plant remains or from products from microbial metabolism. Peptides and various reactive structures could be incoporated in the polymeric materials.

Martin, Haider, and their associates have shown, in several of the studies referenced, how sodium amalgam reduction releases phenolic materials (and in one instance anthraquinone structures) from the polymers. These phenols were the same as those referred to as humic precursors, and many were identical to structures released in the sodium amalgam digests of soil humic substances.

Schnitzer and Ortiz de Serra (1973b) also identified similar sodium amalgam digest products from soil and fungal 'humic' materials, but their yields were low. Permanganate oxidation (Schnitzer *et al.*, 1973) yielded aliphatic mono- and dicarboxylic acids, benzenecarboxylic acids, phenolic acids, and aromatic compounds containing S and N (some of which may have been artefacts). Later, from alkaline cupric oxide digestion studies on 'humic acids' synthesized by *Stachybotrys chartarum*, *S. atra*, and *Epicoccum purpurescens* Schnitzer and Neyroud (1975) suggested that these compounds were, on average, composed of aliphatic materials (38 %), benzenecarboxylic acids (25 %), phenolic structures (21 %), and of materials which released dialkylphthalates (16 %). Such ratios were significantly different from those for soil humic substances. However, this information cannot be regarded as contrary to any thesis which proposes that fungal products contribute significantly to soil humic substances.

Soil humic materials must be considered in terms of highly heterogeneous mixtures formed as the result of numerous chemical and biological synthesis reactions. In addition these materials can be expected to consist of mixtures of old and newly synthesized polymers. For instance, humic substances have been estimated from $^{14}C/^{12}C$ ratio data to have residence times of 100 years in ferrallitic soils (Lobo *et al.*, 1974) and from 5540 years at 18 cm to 9300 years at 30 cm in volcanic soils (Otsuka, 1975). Thus significant changes can be expected to take place during these long residence times. These might involve further biological transformations, and chemical rearrangements and condensation reactions within the polymers. We therefore suggest that it would be highly exceptional to find a fungal humic structure whose composition would be comparable with any soil humic substance.

Thus there is strong evidence to indicate that fungi contribute significantly to the formation of soil humus. Also it would be hard to prove that extracellular enzymatically catalysed synthesis is not important. However, the evidence available at this time for the contribution of purely chemical synthesis is less convincing, and an extensive research programme will be required in order establish its importance in the soil environment.

3.5 PHYSICAL PROPERTIES

Whilst the physical properties of molecules are determined by their chemical composition these properties cannot necessarily be deduced from purely chemical data. This is particularly so in the case of polymeric molecules, and even more so for molecules with poorly defined structural characteristics such as humic acids. Consequently, direct measurements of properties such as molecular size and shape, charge and charge density, and of spectral characteristics are necessary. Knowledge of both the chemical structure and physical properties of humic substances, and the relationships between these is basic to our understanding of the role which they play in the soil.

3.5.1 Spectral Characteristics

Techniques are now available for the accurate recording throughout most of the electromagnetic spectrum of the spectral characteristics of compounds. These range from X-rays, through the ultraviolet, visible and infrared regions, to microwave frequencies. All of these techniques have been applied to humic substances with varying degrees of success and usefulness.

The accurate interpretation of spectral data obtained from polymeric materials is always problematical, and the complex nature of humic substances only serves to increase the difficulties. However, as Felbeck (1965b) pointed out, improvements in techniques of purification and fractionation will increase the usefulness of spectral data. But even so, there are limitations, imposed by the composition and structure of the humic materials, which should always be borne in mind when assessing and interpreting the results obtained from spectral measurements.

Ultraviolet and visible spectra

Humic substances, as has already been pointed out, contain phenolic and various aromatic and possibly aliphatic functional groups which act as chromophores in the visible and ultraviolet regions of the electromagnetic spectrum. However, with very few exceptions the visible and ultraviolet spectra recorded for humic substances tend to be rather featureless. The well defined peaks characteristic of simple organic compounds are absent and even the more diffuse peaks, such as those exhibited by some proteins, are not found. As a

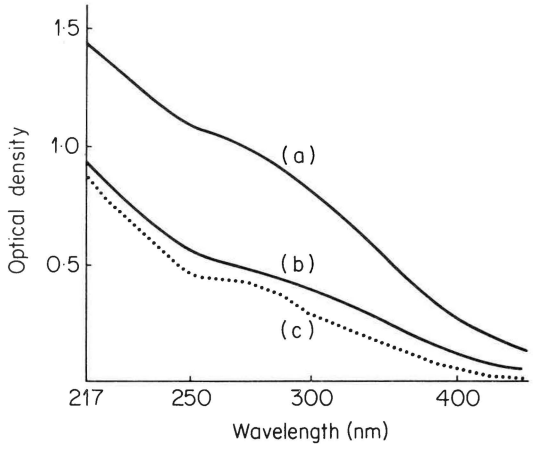


Figure 3.11 Ultraviolet spectra of low (a) and of high (b) molecular weight humic acids (extracted by 0.5N NaOH) from a lateritic podzol and of lignin (c, for comparison). Note the higher extinction coefficient for material (a). (Experimental conditions: sample concentration = 0.2 mg cm^{-3}, cell path length = 2 mm)

rule the spectra show a general increase in absorption with decreasing wavelength (Figure 3.11), although for some materials 'shoulders' or inflections can be detected which perhaps indicate the presence of particularly strong or abundant chromophores.

It is therefore reasonable to assume that humic substances contain numerous chromophores whose absorption bands overlap throughout this spectral region and absorb with growing intensity as the wavelength decreases. It is not possible to observe or measure one particular chromophore, or to derive definitive information with regard to chemical composition or structure. It is possible, however, to utilize the general features of the spectrum, namely the intensity of absorption and the gradient of the spectrum.

The intensity of absorption can be used for quantitative measurements by utilizing the Beer–Lambert law for monochromatic light, which is expressed in the equation

$$A = \log_{10} \frac{I_0}{I} = Kcl$$

where, A is the absorbance or optical density of the solution, I_0 and I are intensity of the incident and transmitted light, respectively, c is the concentration of the absorbing substance, l the path length of the solution, and K a constant which is called the absorptivity or extinction coefficient.

For a single well-defined material K is a constant at a given wavelength. When sample cells of the same path length (e.g. a 1 cm cell) are used the measure-

ments of optical density will be directly proportional to the concentration of solute in solution. It is thus a simple matter to determine solution concentration from calibration curves of known concentrations.

This technique is commonly utilized for quantitative measurements of humic substances since it is simple, accurate, non-destructive, and requires very small amounts of material. Such measurements are usually made in the wavelength band of 400–500 nm, although other wavelengths have been used. However, extinction coefficients, on a weight for weight basis, vary sometimes by as much as a factor of two for humic materials extracted from different soils (Orlov, 1966). Also, even greater variations can occur in the extinction coefficients of samples obtained from the same soil extract. These variations are a function of the molecular weight of the humic acid fractions (Swift *et al.*, 1970, see Figure 3.11). Such differences are not unexpected since the extinction coefficient will obviously be related to factors such as the contents of aromatic and phenolic groups within the humic acid molecules, and these will vary in the fractions of a single extract as well as between extracts from different soils. Thus care should be taken to try to compare only components from different soils subjected to the same isolation and fractionation procedures.

In addition to differences in extinction coefficients humic substances display differences in colour, or hue, ranging from dull grey-brown, through brown itself to a rich red-brown. In terms of spectral characteristics these differences are represented by changes in the gradient of the recorded spectrum. This gradient is most commonly measured between 400 and 600 nm and is expressed as the ratio of the optical densities recorded at these wavelengths. This value is known either as the colour quotient, or the E_4/E_6 ratio, and it is higher for the red-brown materials than for the grey-brown materials. Several attempts have been made to use this ratio as an index of humification (see Schnitzer and Khan, 1972; Flaig *et al.*, 1975 for reviews), i.e. the lower the E_4/E_6 ratio the greater the degree of humification. Although such a hypothesis is not generally accepted it does seem to be true that the more highly condensed and/or the higher molecular weight fractions tend to have low ratio values, whereas the more highly oxidized and/or lower molecular weight fractions tend to give higher ratio values.

Infrared spectra

Infrared spectroscopy is used extensively in chemistry to identify and demonstrate the presence of certain functional groups and to characterize organic compounds. As such it is a useful adjunct to chemical determinations, and in addition it is non-destructive and requires only small amounts of sample. Again the application of this technique to humic substances is somewhat limited by the nature of the material because the presence of numerous infrared active groups leads to overlapping of absorption bands with a consequent lack of resolution. However, certain absorption bands do appear as discrete, or

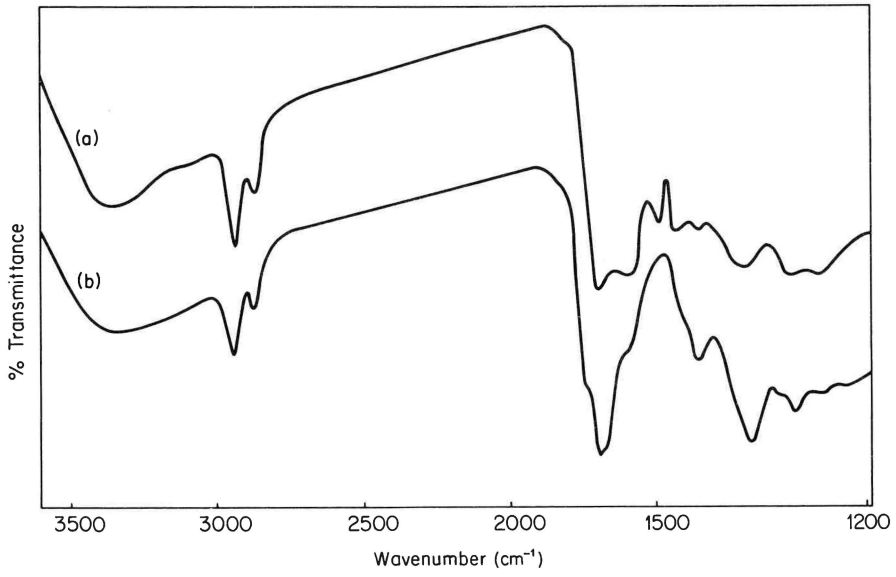


Figure 3.12 Infrared spectra of humic acids: extracted from an organic soil with 0.5N NaOH before (a) and after (b) autoxidation in alkaline solution. Note increased carbonyl (1720 cm^{-1}) and decreased C—H absorption in (b). (From Swift and Posner, 1972).

reasonably well defined peaks and these have proved to be useful for the chemical characterization of humic substances.

Most spectra are recorded by mixing finely ground humic substance with a suitable matrix material (usually *ca.* 1 mg sample with 0.5 g KBr) and pressing the mixture into a transparent disc. Care is necessary to exclude water during this process to prevent spurious results (Theng *et al.*, 1966). A typical humic acid spectrum is shown in Figure 3.12.

The main functional groups revealed by this technique are: hydrogen bonded —OH groups which give a broad absorption band with a maximum around 3400 cm^{-1}, aliphatic C—H giving sharper peaks at 2920 and 2850 cm^{-1}, carboxyl and carbonyl C=O around 1720 cm^{-1} and carboxylate ions ($—COO^-$) at 1610 and 1380 cm^{-1}, C=O conjugated to C=C gives a peak at approximately 1660 cm^{-1}, and a peak at 1610 cm^{-1} is generally thought to be due to aromatic $\rangle C{=}C\langle$. It has been suggested that the peak at 1510 cm^{-1} has similar double-bond origins (Theng and Posner, 1967), although others attribute this peak to the presence of amino compounds (Butler and Ladd, 1969). Attempts have been made to assign other peaks which are sometimes observed, but the validity of such assignments is questionable when they are not supported by direct chemical evidence. Those peaks which are observed and have been assigned tend to support the structural information obtained chemically.

Apart from being used to characterize and compare humic materials obtained from various sources (e.g. Orlov *et al.*, 1962) infrared spectra have proved useful in observing and assessing changes produced by chemical reactions. The processes studied include methylation, acetylation, osazone formation, oxidation, reduction and prolonged exposure to acids and alkalis (Wagner and Stevenson, 1965; Theng and Posner, 1967; Schnitzer and Skinner, 1965a; Dubach *et al.*, 1964; Swift and Posner, 1972). Whilst yielding useful results these studies have also shown that even apparently simple, sharp peaks are usually compound peaks formed by the absorption of several closely related functional groups.

Fourier transform infrared offers a new technique in which the absorption characteristics of aqueous humic suspensions or solutions are digitally recorded over a number of scans (Nagar, 1977), and the spectrum for the water is subtracted out in the computerized set-up. The technique promises to give high resolution quickly over a wide range of the spectrum.

Nuclear magnetic resonance

It is necessary to have samples in solution for successful applications of (proton) ^{1}H n.m.r. because only protons of short relaxation times (*ca* 10^{-3} sec) can be detected by this technique. Thus the properties of the sample under investigation will be influenced by the solvent employed. Inevitably deutrated sodium hydroxide (NaOD) must be used in order to obtain the most comprehensive spectra.

Well differentiated n.m.r. spectra were obtained by Lüdemann *et al.* (1973) for lignins and synthetic and natural humic acids. They estimated the percentages of aromatic protons in the different spectra as: lignin, 30; brown coals, 26–35; synthetic humic acids (from pyrocatechol and hydroquinone), 64 and 78; chernozem and podzol soil humic acids 20 and 44, respectively. Later Lentz *et al.* (1977) used pulsed n.m.r. (100 MHz) and the Fourier transformation technique to compare the spectra for humic acids isolated at pH 4.5, 7 and 14 from the A and B horizons of an Erica podzol. Spectra (run in 0.1N NaOD) recorded the same percentage (34–35) of aromatic protons in the pH 4.5 extracts from the two horizons. Although the aromatic proton content of the B horizon was not influenced by the pH of extraction that of the A horizon was, and the evidence indicated that more aliphatic materials were extracted as the pH was increased in this instance. There was strong evidence for increased numbers of methoxyl groups (peak at 6.2 ppm) for the extracts from the more basic media.

Results by Sciacovelli *et al.* (1977) illustrate how elementary composition and n.m.r. chemical shift data of soil humic materials were influenced by the extractant used. Each of the ten fractions was subjected to infrared and 100 MHz n.m.r. analysis, and it was seen, for instance, that the n.m.r. responses of the dimethyl formamide and dimethylsulphoxide (dipolar aprotic solvents) extracts were very similar, and were less aliphatic than the materials soluble in benzene, dioxane, diethylether, etc.

Further advances in ^{1}H n.m.r. can be expected from applications of a new generation of 300 MHz instruments. However, ^{13}C-Fourier transform n.m.r., which is already widely used for studies on synthetic and bipolymers (such as polysaccharides, proteins, and nucleic acids) may well have a far greater impact on research on humic substances. In principle, for this technique nuclei are excited by applications of pulses of energy and their exponential decay back to the equilibrium state takes the form of a series of sine waves. A Fourier transform, for which an on-line computer is generally employed, relates this decay to a normal n.m.r. spectrum (Levy and Nelson, 1972; Williams and Fleming, 1973). ^{13}C n.m.r. has an additional advantage for studies with humic substances because it should be possible to use aqueous solvents.

Electron spin resonance

Steelink and Tollin (1967) have outlined the principles and applications of electron paramagnetic resonance or e.s.r. for detecting and estimating stable free radicals in humic substances. This spectroscopic technique permits transitions between different energy states to be observed when an external magnetic field induces splitting of the energy levels of a system of unpaired electrons. Spectra can be obtained for humic substances in the solid phase and in solution.

Humic materials provide sharp e.s.r. signals, but these are broadened when paramagnetic metal ions are present. Steelink (see Steelink and Tollin, 1967, for references to the original work) concluded that a polymer containing *ortho*- and *para*-quinhydrones might account for some of the observed properties of his e.s.r. spectra. These, on basification would give rise to semiquinone radical ions as outlined in reaction scheme (3.7).

Atherton *et al.* (1967) reached similar conclusions from their studies with acid boiled humic acids. Their data for the base-solubilized materials indicated that the signals were not from trapped radical species, and the disappearance of these signals on reduction and their reappearance on reexposure to air was considered to be suggestive of the presence of semiquinone-type species. Hyperfine structure or splitting was observed in the spectra.

Senesi *et al.* (1977) observed hyperfine splitting in the e.s.r. spectra of fulvic acids at pH 7.0 only after treatment with H_2O_2, and Senesi and Schnitzer (1977) have also investigated the effects of pH, reaction time, chemical reduction and irradiation on the spectra. The hyperfine splitting was rationalized on the basis of a two-step oxidation of the fulvic acid by H_2O_2; the first step was thought to involve the oxidation of a disubstituted hydroquinone structure to a semiquinone, and the second the oxidation of the semiquinone to the quinone.

Riffaldi and Schnitzer (1972a; 1972b) have quoted free radical contents of the order of 10^{17}–10^{18} spins g^{-1} of humic substances, and these decreased in the order humins > humic acids > fulvic acids. The spins g^{-1} in lignins are generally lower (Steelink and Tollin, 1967, p. 156). Hayes *et al.* (1975a) found that the relative amounts of the radicals in humic and fulvic acids depended on the solvent used for extraction, and their data (Table 3.1) suggested that e.s.r. might

provide a useful means for observing artefact formation when extracting humic substances.

3.5.2 Molecular Weight

One of the fundamental physical properties of any chemical compound is its molecular weight. For a simple organic compound this is a single definable value which can be accurately determined, but with polymeric materials the situation is more complex. In the case of a protein, for instance, which is biosynthesized by a controlled pathway, there may be a single molecular weight value, or at least a series of molecular weights closely grouped around a single value. Many other naturally occurring polymers have relatively wide spreads of molecular weight since the processes involved in their synthesis often give rise to different numbers of monomer units in the molecules. Polymers exhibiting such spreads of molecular weight values are said to be polydisperse, and the degree of the spread, or the polydispersity observed within the system, can vary greatly depending upon the distribution of components of different molecular weights.

When techniques used for determining the molecular weights of well defined compounds are applied to polydisperse systems the results obtained can be used to give average molecular weight values. However, these are often meaningless. Of more interest is the molecular weight distribution, i.e. the range of molecular weights over which the polymer spreads, and the proportion of the whole sample found in any given molecular weight range. A diagrammatic representation of the molecular weight distribution of a polydisperse system is shown in Figure 3.13.

Early attempts to measure the molecular weights of humic acids using simple techniques based on colligative properties gave very low values and confusing

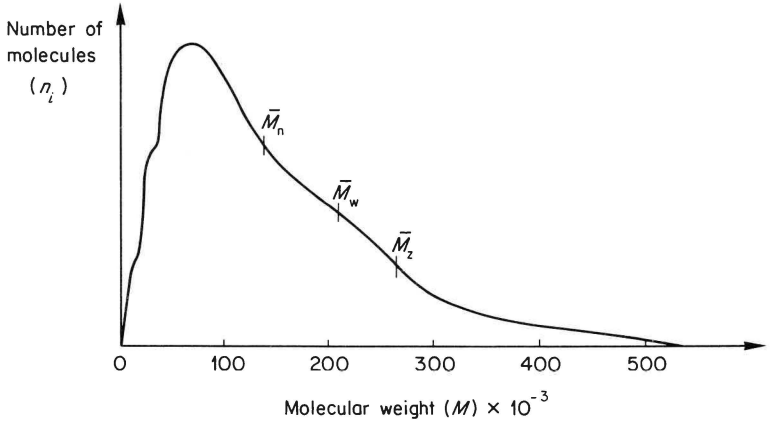


Figure 3.13 Diagrammatic representation of a possible molecular weight distribution, showing M_n, $\bar{M}_w$ and $\bar{M}_z$ values for a humic acid sample after removal of fulvic acid and low molecular weight contaminants

data arising from the effects of polydispersity and of contamination by low molecular weight impurities. Nowadays techniques and mathematical treatments are available to deal with such problems and average molecular weight values can be derived from one or more of the following expressions:

(a) The number-average molecular weight ($\overline{M}_n$), where

$$\overline{M}_n = \frac{\sum n_i M_i}{\sum n_i} \tag{3.32}$$

and the relevant information is obtained from measurements of colligative properties, which depend on the number of solute molecules present;

(b) The weight-average molecular weight ($\overline{M}_w$), given by

$$\overline{M}_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} \tag{3.33}$$

and is influenced more by the weight of individual molecules, rather than their number;

(c) The z-average ($\overline{M}_z$), where

$$\overline{M}_z = \frac{\sum n_i M_i^3}{\sum n_i M_i^2} \tag{3.34}$$

has no simple physical analogy and arises through the mathematical treatment of data from certain experimental techniques, such as sedimentation.

In each case n_i is the number of molecules with a molecular weight M_i. For mono-disperse systems, in which all species have the same molecular weight, $\overline{M}_n = \overline{M}_w = \overline{M}_z$, but for a polydisperse system $\overline{M}_z > \overline{M}_w > \overline{M}_n$. The ratio $\overline{M}_w : \overline{M}_n$ is a useful measure of the polydispersity of a system; the higher the ratio the greater will be the polydispersity or the spread of molecular weights.

The number-average molecular weight ($\overline{M}_n$) is affected equally by all molecules. It is the type of mean we obtain, for instance, when computing the average height of a group of people. In molecular terms it gives a true average. However, in practice the result can be biased towards low molecular weight components (e.g. monomers) which may not be significant by weight but are by number. The weight-average values, on the other hand, tend to emphasize the contributions made by heavier molecules in the system and decrease the effects of the smaller components. Hence this average is probably the most useful for work with humic substances since it helps to overcome the problems associated with the presence of lower molecular weight components or contaminants. An awareness of the different averages in use is important to help explain some of the large discrepancies in the literature values quoted for these materials.

Most of the methods available for molecular weight determination of polymers have been applied to humic substances. However, osmometry, vapour

pressure osmometry, gel chromatography, ultracentrifugation, viscometry, and light scattering have given the most useful results, and we shall limit our consideration to these techniques.

Osmometry

In practical terms osmometry is the only effective technique which can be used to determine the number-average molecular weights of high polymer systems. It requires the use of membranes which permit the passage of solvent but not of solute molecules. The membrane is used to separate a solution (solvent plus polymer) from pure solvent and the resultant difference in chemical potential causes solvent to pass into the solution. In osmometers this flow of solvent is used to create a hydrostatic head whose pressure, when equilibrium is achieved, is equal and opposite to the osmotic pressure across the membrane. Thus the osmotic pressure (π) can be directly measured, and its relationship to molecular weight, derived from van't Hoff's limiting law of osmotic pressure, is given by

$$\underset{c \to 0}{\text{Lt}} \frac{\pi}{c} = \frac{RT}{\overline{M}_n} \tag{3.35}$$

where c is the concentration of solute, R is the gas constant, and T is the temperature (K). The molecular weight $\overline{M}_n$ is determined by extrapolating to zero concentration the values of π determined experimentally at known concentrations.

There are, however, a number of practical problems associated with the technique. Ideally membranes should allow only solvent to pass through, but they tend to be 'leaky' and to allow through some of the lower molecular weight solute species. The older membrane materials, such as cellophane, could exclude only molecules whose molecular weight exceeded 10,000 or even 20,000, but recent advances in membrane technology have produced materials with exclusion limits of 500–1000. Problems of non-ideal membranes can be minimized if equilibration times are rapid, or by extrapolating results back to zero time.

The method also loses sensitivity for high molecular weight materials. Equation (3.35) shows that when $\overline{M}_n$ is very large, and the concentration c is small as is necessary, π will be small. Thus it becomes difficult to measure π accurately for molecular weight values of approximately 200,000 and higher.

A further complication is introduced by the fact that humic substances are polyelectrolytes, and are macro-ionic in nature. Because of this the polymer solution on one side of the membrane will contain simple counterions as well as polymer molecules. Since humic acid polymers are highly charged the number of counterions in the solution will be orders of magnitude greater than the number of polymer molecules. Therefore, because osmotic pressure is a colligative property dependent solely on the number of species in solution, each counterion will be as effective as a polymer molecule in producing an osmotic effect. Any molecular weight value measured under such conditions will be meaningless.

This effect can be countered by adding to the system a simple electrolyte which is able to pass freely through the membrane. However, this introduces further problems arising from Donnan effects (see Chapter 5). Because of these the total concentration of simple electrolyte ions will be greater on the polyelectrolyte side of the membrane and will again lead to erroneous values. These effects can be minimized to negligible proportions if sufficient electrolyte is added to the system (Tanford, 1961, p. 225).

Where the required attention has been given to experimental technique and operating conditions osmotic pressure measurements have yielded useful results. Wright *et al.* (1958) using a solution of dialysed humic acid extract obtained from a podzol B horizon reported a mean molecular weight value of 50,800. Wake and Posner (1967) applied osmometry to measure the molecular weights of humic acid extracts fractionated by ultrafiltration through newly developed semipermeable membranes of different pore sizes. The values obtained for isolated fractions varied from 2000 up to approximately 34,000. They also showed that although the mean molecular weights for two whole extracts were very similar, the molecular weight distributions were considerably different. By use of a similar fractionation technique on a different humic acid extract Swift *et al.* (1970) determined $\overline{M}_n$ values ranging from 1500 to 65,000. When these fractions were passed down a gel chromatography column they were eluted at the positions predicted by the osmotic pressure molecular weight measurements.

The difference in chemical potential between a solvent and solution, resulting from the presence of a dissolved polymer, will also give rise to a difference in vapour pressures above the two liquids (in addition to an osmostic pressure between them if they are separated by a semipermeable membrane). In general, because this vapour pressure is much less than the osmotic pressure, measurement of the latter is preferred. However, improvements in design, and the commercial availability of vapour pressure osmometers has led to a renewed interest in the application of this rapid and relatively simple technique.

When drops of solvent and of solution are placed side by side in a closed container, maintained at constant temperature and saturated with solvent vapour, the solvent vapour will condense in the solution because the latter has the lower vapour pressure. As a result latent heat is discharged by the solvent condensing into the solution and the temperature of the solution will rise to a quasi-steady state value. If the instrument is accurately calibrated and care is taken to standardise variables such as drop size and time of measurement it can be shown, by a combination of the Clausius–Clapeyron equation and Raoult's law, that

$$\frac{W_2}{M_2}\frac{W_1}{M_1} = \text{constant} \times \Delta T \tag{3.36}$$

where W_1 = weight of solvent in the solution, M_1 = molecular weight of solvent, W_2 = weight of solute in the solution, M_2 = molecular weight of solute

and ΔT = temperature increase of solution over solute.

A major improvement in instrumentation has been brought about by the use of thermistors whose resistance (R) changes with the change in temperature. Thus in practice, ΔT is measured as ΔR, i.e. the difference in resistance between sample and reference thermistors.

This technique is most effective in non-aqueous solvents or in aqueous systems where electrolyte is absent. However, Hansen and Schnitzer (1969c) have applied it to studies of acid soluble fulvic materials from a podzol B_h horizon where difficulties were experienced due to polyelectrolyte properties. They attempted to correct the molecular weight measurements by allowing for the dissociation of acidic functional groups. Although molecular weight values up to 3600 were recorded, most of the extract had values of the order of 1000 and less, which is rather low for polymeric systems. However, this particular humic fraction is thought to consist largely of low molecular weight components which would greatly influence the colligative properties and give low values for $\overline{M}_n$. Only values of $\overline{M}_n$ can be obtained in practice by this technique, and apart from their intrinsic merits such data are necessary to estimate the degree of polydispersity of samples from $\overline{M}_w/\overline{M}_n$ values.

Gel chromatography

Gel chromatography is a simple, relatively inexpensive, and very effective technique for studying polymers. It can be used as a method for separation, purification and fractionation as well as for determinations of molecular weights and molecular weight distributions of polymer systems. A review of the principles and applications of the technique is provided by Fischer (1969).

Although inorganic materials, such as porous glass beads, have been used, the gels most commonly employed consist of cross-linked polymers (e.g. polysaccharides, polystyrene, and polyamides) in the form of small granules. The gel structure is perfused by a system of pores and the size of these is determined by the degree of cross-linking in the polymer. These pores enable the gel to act as a chromatographic medium giving separations based on differences in molecular sizes. In practice the gel beads are swollen in an appropriate solvent (usually an aqueous salt solution for polymers of biological origin) and are packed into a column to form a stationary phase. When a solution, containing a mixture of molecules of varying sizes, is applied to the top of the column and eluted with solvent those molecules which cannot enter the pores in the beads will pass between them and will be eluted first from the column. Molecules smaller than the pore sizes of the gel will enter the pores and their passage through the column will be retarded. The extent to which this occurs will depend on the actual size and shape of the molecule, but the net result is that the solute molecules are eluted from the column in order of decreasing molecular size and, for a given polymer, decreasing molecular weight. This process is shown diagramatically in Figure 3.14.

When small samples are applied to the columns, solutes with discrete mole-

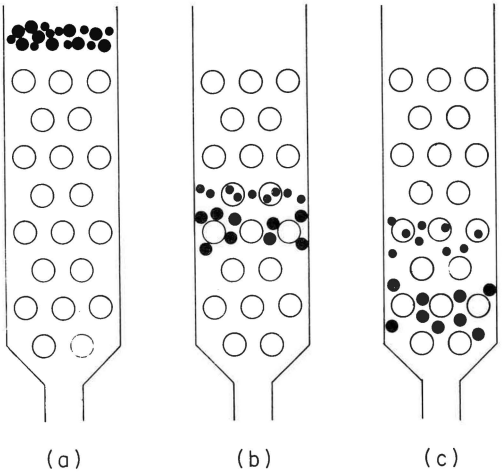


Figure 3.14 Diagrammatic representation of the gel chromatography process showing partial retention (b and c) of smaller molecules by the gel

cular weights are eluted as well defined peaks. The position at which the peak of a given solute is eluted can be characterized by calculating the partition coefficients denoted by K_{av} or K_d values where

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \tag{3.37}$$

Here V_0 is the void volume, or the volume of solvent outside the gel beads forming the bed, and it is also the volume in which a totally excluded solute will be eluted. V_e is the volume required to elute the solute being examined, and V_t is the total volume of the column. The value K_d is more accurate because it eliminates the volume of gel which is included in V_t but is not strictly a part of the inner phase; thus

$$K_d = \frac{V_e - V_0}{V_t - V_0 - V_g} \tag{3.38}$$

where V_g is the volume of the gel. For highly swelling gels the difference between K_{av} and K_d is not significant. However, the values will be different when the proportion of the total volume occupied by the actual gel matrix increases, for instance with glass beads or with only slightly swelling gels.

Ackers (1964) interpreted gel chromatography on the basis of steric and frictional resistance to the diffusion of solute in the gel pores, and derived a relationship between K_d and the Stokes' radius a of the solute where consideration was given to r, the effective radius of the gel pores (assumed to be cylindrical). A value for r can be obtained for a given gel by measuring the K_d for a solute of known Stokes radius. When K_d values are plotted against the logarithms of

corresponding values of r sigmoid curves with extended linear regions are obtained which can be expressed as

$$K_d = -K_1 \log\left(\frac{a}{r}\right) + K_2 \tag{3.39}$$

where K_1 and K_2 are constants.

For any solute a is proportional to some fractional power x of the molecular weight M. This fractional power depends on the shape of the molecule but it will be the same for any series of similar molecules. Assuming that $a = M^x$ for a particular type of molecule, then for any series of molecules of this type

$$K_d = -k_1 \log M^x/r + k_2 \tag{3.40}$$

which simplifies to

$$K_d = -b \log M + c \tag{3.41}$$

where b and c are constants. Thus, it can be seen that there is a relationship between the molecular weight and the position of elution of a component, and that once calibrated for a particular group of polymers, gel chromatography provides a rapid and facile method for estimating molecular weights. Gels are available which operate over different molecular weight ranges and have different exclusion limits. By utilizing a range of gels it is possible to determine molecular weight values ranging from several thousands to millions.

This technique has been extensively and successfully applied to studies of humic substances. A number of problems are, however, encountered which, if they are not overcome, can invalidate the results. For instance, the gel material should be inert to the solute molecules so that there are no chemical or physical interactions between gel and solute. When any adsorption of the applied polymer molecules by the gel takes place the observed retention by the column is not solely caused by penetration into the pores and the resulting separation cannot be entirely attributed to molecular weight differences. Because of their chemical composition humic substances tend to be readily adsorbed.

A further problem arises as the result of charge interactions between residual charged groups on the gel and those on the humic substances leading to attractive or repulsive forces between the gel and charged humic substances. If not suppressed these charges interfere with the separation which again would not take place solely on the basis of molecular size differences.

Swift and Posner (1971) showed that such problems can be largely overcome by careful selection of the gel matrix and by the use of appropriate buffer solutions. Use of a buffer containing a large organic cation such as tris[2-amino-2(hydroxymethyl)propane-1,3-diol], or similar compounds is recommended. Even when such procedures are used there is some indication that a small amount of interaction between gel and solute can still take place, particularly in the cases of the very high molecular weight, less soluble humic acid fractions.

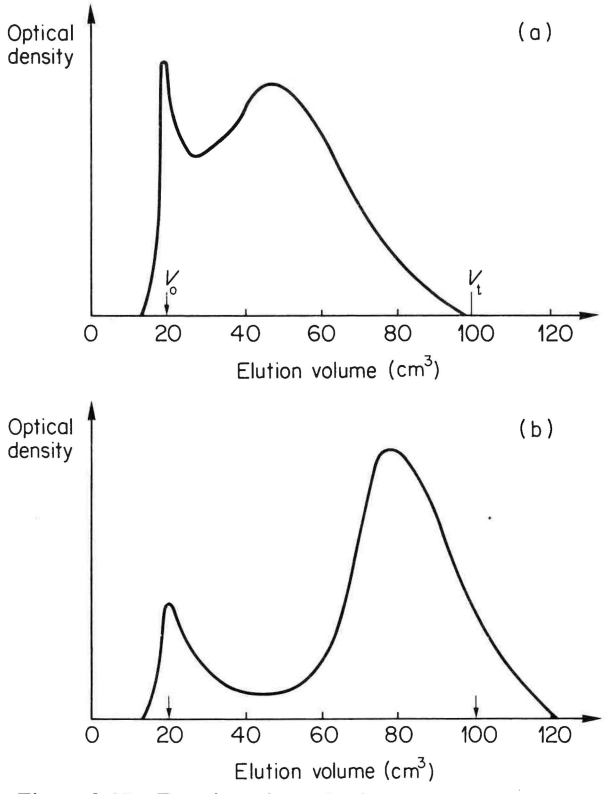


Figure 3.15 Fractionation of a humic acid by gel chromatography on Biogel P-150 (a) and Biogel P-300 (b). (These gels have nominal molecular weight exclusion limits of 150×10^3 and 300×10^3, respectively)

Despite these handicaps gel chromatography has proved to be a particularly useful technique for the purification, fractionation, and determination of the molecular weight of humic substances.

Typical elution patterns obtained by means of the gel chromatography of humic acid extracts are shown in Figure 3.15. For the system illustrated a peak consisting of excluded material appears at the void volume V_0, and the remainder is spread as a diffuse peak over the working range of the column. The proportions found in each of these peaks will depend upon the molecular weight distribution of extracts and the exclusion limit of the gel. Thus if a gel with a higher exclusion limit is used the size of the retained peak will be increased at the expense of the V_0 peak. Elution patterns such as these (e.g. Dubach *et al.*, 1964; Swift and Posner, 1971) confirm the polydisperse nature of humic substances and show that they cover a wide range of molecular weight values. Cameron *et al.* (1972b) separated by gel chromatography humic acid fractions ranging in molecular weight from 2000 to 1,500,000, and showed that the most abundant

portion of the molecular weight distribution for a sodium hydroxide extracted humic acid was around 100,000.

When selecting gels for determinations of molecular weight values and distributions, consideration should be given to the molecular weight ranges over which the gels operate and in particular to their upper exclusion limit. The manufacturers of gels supply figures for these properties, and such values have been quoted extensively when reporting values for the gel chromatography of humic substances. However, the manufacturers values are obtained by calibrating with proteins or polysaccharides of known molecular weights. It is unlikely that humic acid polymers would have the same configurations or dimensions in solution as polysaccharides or proteins of similar molecular weights, and hence would behave differently in gel chromatography. Thus manufacturer calibrations are not likely to be valid for humic substances. This problem can be overcome by calibrating the gel materials with humic acid fractions of known molecular weights determined by osmometry or by ultracentrifugation on well fractionated samples. Such fractions have been used (Swift *et al.*, 1970; Cameron *et al.*, 1972a) to calibrate a series of gel chromatography materials and these calibrations were compared with those obtained using proteins or polysaccharides. In general, the calibrations from humic acid fractions differed significantly from those based on proteins which tend to have tightly-coiled, globular molecular configurations. However, there was reasonable agreement with some calibrations obtained using polysaccharides which tend to have less compact, randomly-coiled molecular configurations.

The technique of gel filtration is contributing significantly to the problem of isolating humic fractions of low polydispersity, and it has considerable potential for estimations of molecular weight values of the less polydisperse isolates. Such molecular weight determinations might be simplified, as suggested by Cameron *et al.* (1972a), by establishing a relationship between the molecular weights of humic substances and a selection of proteins or polysaccharides. This would permit the uses of these latter materials as secondary standards.

Ultracentrifugation

Sedimentation of finely divided solids from suspensions is achieved by normal centrifugation techniques. However, in order to sediment molecules from solution it is necessary to use the ultracentrifuge which, by its high rotational speeds, generates very strong force fields.

The usual design of analytical ultracentrifuge consists of a solid rotor which rotates about a central axis and contains a small sample cell, shaped like a segment of the rotor. When the cell is positioned approximately 65 mm from the axis or rotation and spun at 60,000 r.p.m. the contents experience a force of 250,000 times that of gravity.

If a dilute solution of macromolecules is spun in the cell the solute molecules will gradually sediment to the bottom of the cell. This process is shown dia-

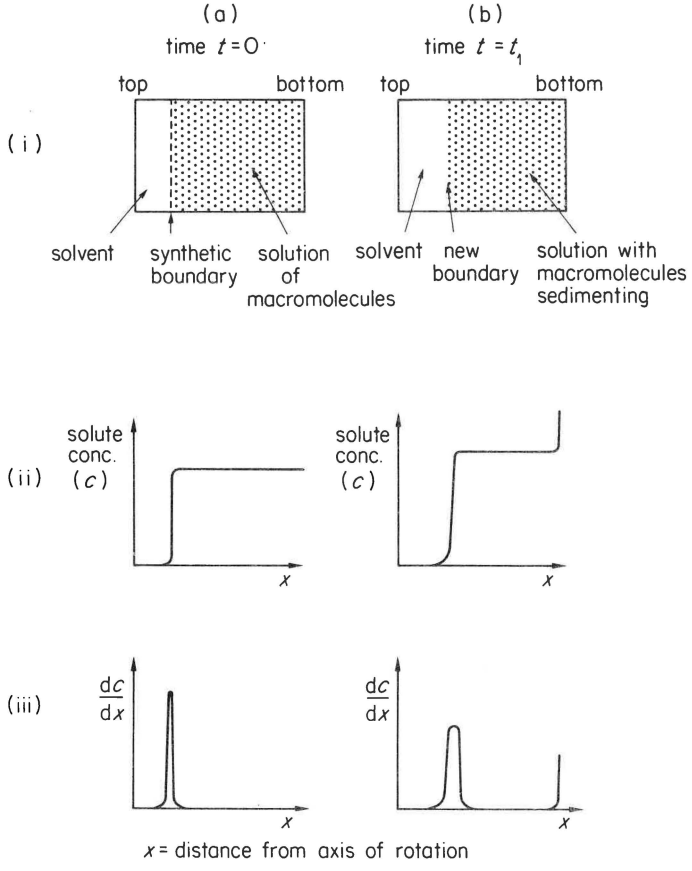


Figure 3.16 Diagrammatic representation of the ultracentrifugation process (using Schlieren optics) at the commencement (a) and during a run (b) showing (i) the distribution of cell contents, (ii) the plot of solute concentrations in the cell, and (iii) the peaks given by Schlieren optics as the derivatives of (ii)

grammatically in Figure 3.16. Several optical systems can be used to measure the changes in concentration that occur as the solute moves down through the cell. Many instruments are fitted with a version of the Schlieren optical system which measures differences in refractive index arising from concentration changes in the cell. For this system the change in concentration is given in a differential form so that the boundary between the pure solvent and the solution, formed by the sedimentation of the solute, appears as a peak. The rate of sedimentation of the solute can be determined by following the movement of this peak during an experimental run.

Measurements of optical densities provide the simplest technique for observing changes in concentration in coloured or ultraviolet-absorbing solutions.

For these the cell is scanned by a beam and the data can be presented as concentration, as a function of distance along the cell, or as the appropriate derivatives. The method is capable of a high degree of refinement, and the sensitivity of ultraviolet or visible absorption techniques allows very low solute concentrations to be used. Higher solution concentrations, of the order of one per cent or more of polymer, are generally required for the Schlieren system. Since molecular weight values are obtained by extrapolating measurements back to zero concentration it can be an advantage to use the absorption systems in which data for low concentration levels can be obtained.

Humic materials have a very intense coloration, and absorb strongly in the visible and ultraviolet regions of the spectrum (see Figure 3.11). This causes some problems in the use of traditional optical systems in the ultracentrifuge. The Schlieren system operates most effectively at higher concentrations, but the transmission of light through humic acid solutions at the desired concentrations is very low. Thus it is necessary to lower the concentration in order to transmit sufficient light for photographic recordings to be made. However, if the concentration is greatly reduced the Schlieren peak becomes too small and ill-defined for accurate measurements. Cameron *et al.* (1972b), using fast film, reported an upper concentration limit of 2.4 g dm^{-3} and a lower limit of 0.6 g dm^{-3}. The upper limit could probably be increased by use of a higher intensity light source of longer exposure times; indeed Piret *et al.* (1960) reported measurements of solutions containing 7.3 g dm^{-3} of humic acid.

The strong light absorption properties of humic substances would suggest that they could be effectively analysed by absorption optics. But because the optical extinction coefficients of humic acids vary as a function of molecular weight (Swift *et al.*, 1970), the optical density is not directly related to concentration for the highly polydisperse material. This difficulty can be overcome by applying suitable corrections where unfractionated extracts are used, as for instance in equilibrium ultracentrifugation experiments. However, when sedimentation coefficients are required, only well fractionated samples should be used in the ultracentrifuge. In this way variations in optical extinction coefficients will be small and may not require correction.

An optical system based on the Rayleigh interferometer technique can also be used to overcome the problem of variations in optical extinction coefficient in samples which exhibit a large molecular weight range. This system reponds to changes in refractive index which occur as solute concentration varies. It can be used at low solute concentrations, and since the refractive index increment per unit weight is not likely to vary with molecular weight among chemically closely related species, it can be used equally effectively on polydisperse or fractionated samples. Posner and Creeth (1972) have successfully used interferometry for their studies on unfractionated humic acids by equilibrium ultracentrifugation.

There are two principal methods which can be used to calculate molecular weight values from data obtained by observing concentration changes in the cell during an experimental run. The most common involves the use of values for

sedimentation and diffusion coefficients. The sedimentation coefficient, s, represents the velocity of solute molecules divided by the centrifugal field so that

$$s = \frac{1}{x\omega^2} \cdot \frac{dx}{dt} = \frac{d \log_e x}{\omega^2 \, dt} \tag{3.42}$$

where x is the distance of the solute/solution boundary (e.g. Schlieren peak) from the axis of rotation, ω the angular velocity and t the time. Thus a plot of $\log_e x$ against time should give a straight line and s can be obtained from its slope. As long as the density of the solute exceeds that of the solvent, which is generally the case, the solute will move to the bottom of the cell under the influence of the centrifugal force. This movement will be opposed by diffusion processes which will tend to redistribute the solute evenly through the cell. Thus the diffusion coefficient, D, of the solute must be determined to allow calculation of the molecular weight. This can be done in the ultracentrifuge, or preferably by an independent method. When s and D are used in the same calculation it is essential that they be determined at similar temperatures. It is also usual to extrapolate the results obtained to zero concentration of solute, and to correct to standard conditions; i.e. for water as solvent at 20 °C. These values are often written as $s^\circ_{20}w$, and $D^\circ_{20}w$. We shall assume here that the symbols s and D are the appropriate corrected values.

The molecular weight can be calculated from the Svedberg equation

$$M = \frac{RTs}{(1 - \bar{v}\rho)D} \tag{3.43}$$

where M is the molecular weight, R the gas constant, T the temperature (K), $\bar{v}$ the partial specific volume of the solute, and ρ the solution density. The values of $\bar{v}$ and ρ can be determined by standard techniques using pycnometers.

A second method, which does not require measurement of the diffusion coefficient, calculates molecular weight values by using equilibrium ultracentrifugation. In this technique the rotor is run for a long time at relatively low speeds until sedimentation is equal and opposite to diffusion, and no net movement of solute occurs. It can then be shown that

$$M = \frac{2RT \, d \log_e c}{(1 - \bar{v}\rho)\omega^2 \, dx^2} = \frac{RT}{(1 - \bar{v}\rho)} \cdot \frac{dc/dx}{\omega^2 xc} \tag{3.44}$$

where c is the solute concentration at distance x from the axis of rotation.

The problems caused by the polyelectrolyte nature and the polydispersity of humic substances must again be considered before meaningful results can be obtained from ultracentrifugation experiments. Humic acid molecules in solution carry a high negative charge which is usually balanced by suitable cations and these will be dissociated to give a diffuse, positively charged atmosphere around the negatively charged polymer molecule. The system will then

behave as predicted by diffuse double layer theory (Chapter 5), and the polymer molecules will tend to repel each other. This behaviour will have a pronounced effect on ultracentrifugation experiments. The intermolecular repulsion will give anomalously high diffusion values, and in addition, during centrifugation the large macromolecules will tend to sediment faster than their counterions causing electrostatic drag and a lower value for the measured sedimentation coefficient. From equation (3.43) it can be seen that high D and low s values will result in anomalously low molecular weight values. These effects can be readily overcome by using an excess of neutral electrolyte; 0.1 to 0.2M additions of salt are usually sufficient. The added salt can itself produce secondary charge effects due to the difference in sedimentation behaviour between anion and cation, but this can be avoided by using an electrolyte, such as potassium chloride, where there is negligible difference in the behaviour of the two ions.

The importance of suppressing charge effects is evident from results obtained from ultracentrifugation studies on humic substances carried out by Flaig and Beutelspacher (1968). When sedimentation and diffusion coefficients were measured in water, a mean molecular weight value of 2050 was obtained. Addition of 0.2M sodium chloride suppressed the charge effects and the same humic material then gave a more realistic mean molecular weight value of 77,000.

The problem presented by the polydispersity of humic substances is more difficult to overcome. It must be remembered that in highly polydisperse systems numerous molecules of different sizes and different diffusion coefficients will be sedimenting at different velocities. This makes it impossible to obtain a well-defined sedimenting boundary. Even when such boundaries are formed artificially, as in a synthetic-boundary cell (see Figure 3.16), they will spread rapidly and disappear. Although short-lived Schlieren peaks can be obtained in this way these are highly asymmetric, and the values derived from them lead to erroneous results. The most effective way to deal with the problem is to decrease the polydispersity, by fractionation procedures, to a level where meaningful results can be obtained. Suitable gels and membranes are now available which allow fractionation of humic substances to acceptable levels of polydispersity.

Prior to the availability of the modern fractionation procedures some interesting applications of the sedimentation velocity technique were used in studies on humic substances. Stevenson *et al.* (1953) used a dialysed but unfractionated sodium pyrophosphate extract and obtained a weight-average molecular weight value of 53,000. The movement of the sedimentation boundary was followed by using a photodensitometer in conjunction with direct photographs of the cell contents; essentially an optical absorption technique. Later Piret *et al.* (1960) calculated a mean molecular weight value of 25,000 for a sodium hydroxide extract. In this study the diffusion coefficient was not measured directly but calculated from frictional data obtained from viscometric work. Charge effects were suppressed, but the spreading of the Schlieren peak due to polydispersity was considerable. Even though this peak remained stable long enough for measurements to be made, the asymmetry effects, referred to above, may have introduced considerable error into the molecular weight calculation.

Particular attention was given to decreasing polydispersity by Cameron *et al.* (1972b). They investigated molecular weight values by the ultracentrifugation of humic materials which had been extensively fractionated by means of gel chromatography and pressure filtration techniques. The processes involved were time consuming but led to the isolation of materials with relatively narrow limits of polydispersity. Fractions were taken at intervals over much of the molecular weight range of the parent material, and each was individually examined in the ultracentrifuge by the sedimentation velocity technique. Diffusion coefficients were measured independently and the molecular weight values calculated ranged from 2.3×10^3 to 1.4×10^6. This work indicates that more reliable and informative data can be obtained by working with fractions covering only narrow molecular weight ranges and that the averages obtained are more truly representative of the material being examined. For instance, a mean molecular weight value of the order of 5×10^4 for unfractionated material might be composed of molecules ranging from say 2×10^3 to 1.5×10^6, but in the case of the fractionated material the same value might refer to material with a molecular weight spread of the order of 4.5×10^4 to 5.5×10^4.

Useful results can be obtained for polydisperse materials if equilibrium ultracentrifugation is used. The technique requires a very high standard of instrument performance, sophisticated optics, and a prolonged running time. Posner and Creeth (1972) obtained $\overline{M}_n$ and $\overline{M}_w$ values for unfractionated humic acids and were able to estimate polydispersity from the $\overline{M}_n/\overline{M}_w$ ratio. When high molecular weight components sediment to the bottom of the cell at the rotor speed used they are not included in the calculations and the results obtained will not represent the true mean value. Thus even with this technique some prior fractionation is desirable.

Ultracentrifugation can also be used to determine molecular weight distributions by using a density gradient technique. For this a density gradient is established in the cell by use of suitable solutions so the buoyancy of the solution medium increases along the length of the cell. A point is reached for each solute molecule when the term $(1 - \bar{v}\rho)$, in equation (3.43), equals zero and it ceases to sediment. When all of the solute species have reached equilibrium the distribution of solute within the cell can be determined (e.g. colorimetrically) and the molecular weight distribution can be calculated. The technique has the added advantage that less expensive, preparative ultracentrifuges with swing-out heads can be used.

Gradients are usually formed by using solutions of inorganic salts, such as rubidium chloride or caesium chloride, or of sucrose. In applying the technique to humic acid studies Cameron and Posner (1974) used a D_2O/H_2O density gradient to determine $\overline{M}_n$, $\overline{M}_w$, and $\overline{M}_z$ values for a number of extracts. They reported the following molecular-weight values for an unfractionated sodium pyrophosphate extracted humic acid from a Fen peat: $\overline{M}_n = 13.5 \times 10^3$, $\overline{M}_w = 216 \times 10^3$ and $\overline{M}_z = 1100 \times 10^3$. These results provide striking evidence for the extreme polydispersity of humic substances and of the usefulness of the density gradient procedure when working with such materials.

Viscometry

Viscosity is a measure of the frictional resistance that a flowing liquid offers to an applied shearing force. When fluid flows past a stationary phase the successive 'layers' extending from the stationary surface will move with increasing velocities. Velocity is described as Newtonian when the shearing force, τ, per unit between two parallel planes of liquid in motion relative to each other is proportional to the velocity gradient, dv/dx, between the planes and

$$\tau = \eta \, dv/dx \tag{3.45}$$

where η is the coefficient of viscosity. Newtonian viscosity is constant and independent of the rate of shear.

We will be interested here in deviations arising from the relative viscosity η_{rel} sometimes called the viscosity ratio as recommended by the International Union of Pure and Applied Chemistry (IUPAC), which is defined as the solution viscosity, η, divided by that of the pure solvent, η_0. These are conveniently measured by means of capillary viscometers such as the Ostwald–Fenske and the Ubbelohde instruments described in physical chemistry texts. These instruments are simple to operate and have almost invariably been used in viscometric studies on soil humic substances. Their main disadvantage is that the rate of shear varies from zero at the centre of the capillary to a maximum at the wall. Thus it should be remembered that anisotropic particles would assume various states of orientation over the cross-section of the viscometer, and although reproducible results would be obtained these would have little theoretical significance. For such systems rotational methods, which use the concentric cylinder, and the cone and plate techniques are particularly useful. The reader is referred to colloid chemistry texts for descriptions of the design and the use of such instruments, and only brief reference will be made here to the first of these methods. In this a thin film of liquid is placed between the concentric cylinders, the outer cylinder is rotated at a constant rate, and the shear stress is measured by considerations of the deflection of the inner cylinder which is suspended by a torsion wire.

Where an Ostwald-type viscometer is used the pressure at any instant which drives the liquid through the capillary will be proportional to its density. Thus

$$\eta = k\rho t \tag{3.46}$$

where k is the viscometer constant, ρ is the density of the liquid, and t is the time of flow. For two different liquids

$$\eta_1/\eta_2 = \frac{\rho_1 t_1}{\rho_2 t_2} \tag{3.47}.$$

From this relationship it can be seen that at infinite dilution, where the densities of solvent and of solution are essentially the same, the viscosity ratios will be equal to those of the times of flow.

The specific viscosity, η_{sp}, is a measure of the relative increase in the viscosity of the solution over that of the solvent alone. Thus

$$\eta_{sp} = \frac{\eta_c - \eta_0}{\eta_0} \tag{3.48}$$

where η_0 is the viscosity of the solvent and η_c is that of the solution of concentration c. The specific viscosity divided by concentration η_{sp}/c, is known as the reduced viscosity (or viscosity number in IUPAC nomenclature), and the intrinsic viscosity, $[\eta]$, or limiting viscosity number (IUPAC), is defined as

$$[\eta] = (\eta_{sp}/c)_{c \to 0} \tag{3.49}$$

Values for $[\eta]$ can be obtained from extrapolations to zero concentrations of the plots of η_{sp}/c versus c as is implicit in the relationship

$$\eta_{sp}/c = [\eta] + k'[\eta]^2 c \tag{3.50}$$

or by the alternative relationship

$$\log \eta_r/c = [\eta] - k''[\eta]^2 c \tag{3.51}$$

where k' and k'' are constants, and c is expressed in g dl^{-1}.

The intrinsic viscosity is related to molecular weight by

$$[\eta] = KM^{\alpha} \tag{3.52}$$

in which the two empirical coefficients K and α are constants for a given polymer in a given solvent over a range of molecular weights. Equation (3.52) transforms to

$$\log [\eta] = \log K + \alpha \log M \tag{3.53}$$

which provides values for K and α from the plot of $\log [\eta]$ versus $\log M$. Obviously in order to use this method it is necessary to obtain at least two molecular weight values by independent methods for carefully fractionated monodisperse fractions of the polmer.

Both K and α are functions of the solvent as well as of the polymer. This empirical relation between viscosity and molecular weight must, however, not be used indiscriminately as it is most suitable for studies with linear polymers.

Viscosity data can be used to find viscosity-average molecular weight values ($\overline{M}_v$). Rafikov *et al.* (1964) give details of the mathematical manipulations required.

Viscosity is markedly influenced by changes in polymer conformations. We have repeatedly referred to the fact that when a polyelectrolyte solution is diluted increased ionization takes place and polymer expansion is enhanced as the result of repulsion by similarly charged functional groups along the polymer chain. When very high extensions are reached the effect is often reversed, and a characteristic convex curve is obtained for the η_{sp} vs. c plot when measurements are extended to very low concentrations. When dealing with gel filtration and ultracentrifugation, the addition of salt depresses the ionization effects in the

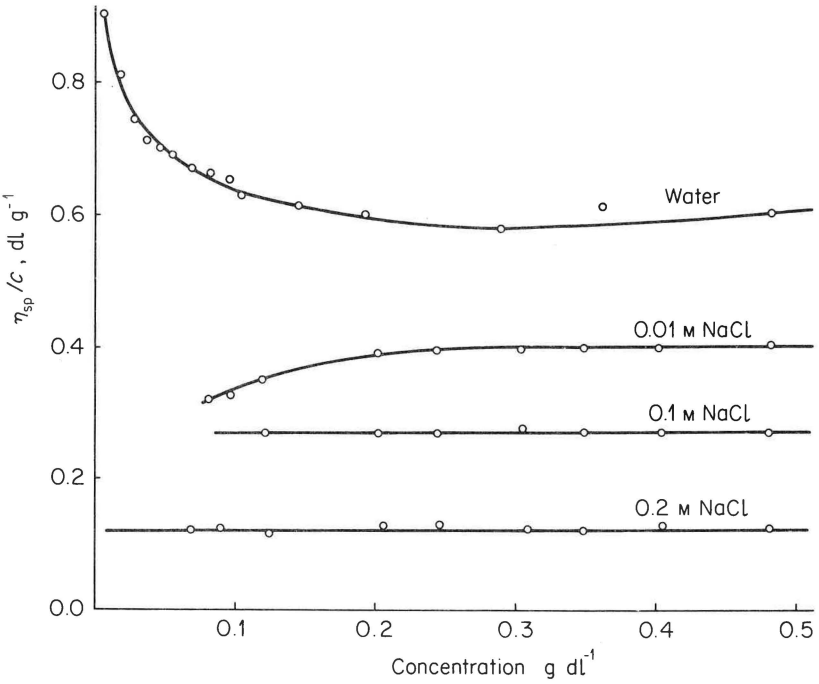


Figure 3.17 Plot of reduced viscosity (η_{sp}/c) versus concentration for a sodium humate showing the effects of added salts. (After Smith and Lorimer, 1964.) Reproduced by permission of the Agricultural Institute of Canada

polyelectrolyte. Thus it has been possible to obtain meanigful results from viscosity measurements in many instances where the addition of an appropriate amount of salt caused the polyelectrolytes to behave like neutral polymer molecules.

The influence of electrolytes on the viscometric properties of humic substances is well recognized (Flaig *et al.*, 1975). Mukherjee and Lahiri (1956) observed the characteristic increases in the η_{sp}/c values of dilute aqueous solutions of K^+-humate extracted from an air-oxidized low rank coal. These values were depressed in the presence of KCl at concentrations of $4–8 \times 10^{-3}$ g dl^{-1}. Data from Smith and Lorimer (1964) presented in Figure 3.17, show how the addition of salt influenced viscosity values in the case of humic acid materials extracted from a sphagnum peat. Piret *et al.* (1960) also observed a concave upwards relationship for the η_{sp}/c vs. c plot at low concentrations of a peat Na^+-humate in water. Although their viscosity data extended to very low concentrations of solute there was no evidence for diminished viscosity at the lowest dilutions measured. However, they observed a straight line relationship with a positive slope when measurements were carried out in the presence of 0.26M NaCl. When the NaCl concentration was only 0.1M a small convex upwards 'hump' was observed with a maximum at a polymer concentration of *ca.* 0.2 g dl^{-1}. This

indicates that 0.1M NaCl was insufficient to completely depress the ionization of humic substances at very low concentrations.

Molecular weight data for humic substances, which have been reported from viscosity measurements up to the present time, can only be regarded as relative and not as absolute values because the materials used were invariably highly polydisperse. We suggest that it might be possible to derive more meaningful data from viscosity measurements by working with well fractionated samples and by careful additions of salt in order to derive $[\eta]$ values from plots of η_{sp}/c vs. c (equation 3.50). It will be necessary to observe carefully whether or not the values of K and α change from sample to sample. If they do not the viscosimetric method could be used as an inexpensive method for determining the molecular weights of samples and also for measurements of polydispersity.

The average molecular weight data, from viscometric measurements on humic acid by Piret *et al.* (1960) and Visser (1964) were calculated from equations designed for synthetic polymers of known structures and conformations. Thus the values of 5×10^4 and 36×10^3 quoted by the authors were only regarded as order of magnitude approximations by them because of the constraints implicit in the equations used.

Plots of η_{sp}/c vs. c gave Chen and Schnitzer (1976) straight line relationships for humic acid at pH 7.0, and for fulvic acid at pH values of 1.0 and 1.5 only. Salts were not added. It is surprising that the η_{sp}/c value did not change as the pH 7.0 humic acid sample was diluted. Considerable dissociation of the polymer carboxyls would be expected at this pH value to give increasing polymer expansion (and viscosities, as was observed at pH 8.5) on dilution. On the other hand it is equally difficult to explain a change in slope which was observed for a similar plot for fulvic acid at pH 2.0. This change might indicate some ionization, and only strong acids would be dissociated at such low pH values. Unionized fulvic and humic acids would be expected to behave like neutral polymers in viscosity measurements, even in the absence of additions of salt.

Light scattering

When light passes through a dilute polymer solution some will be scattered and from the intensity of the scattered light the molecular weight and shape of the polymer molecule can be calculated. However, it is important that the scattering of light be due only to the polymer and not to dust or other impurities in the system. Tanford (1961) and Billmeyer (1971) give more details about the principles of light scattering.

The fluorescence of humic substances gives rise to problems where light scattering studies are employed because it can cause considerable amounts of light to be detected as if it were scattered light. This problem can be overcome by judicious use of filters or monochromators. But, as yet, there is no evidence to indicate that such precautions have been taken when measurements were made on humic substances and hence the interpretations given for the data may not

be highly accurate. Orlov and Gorskova (1965) report weight-average molecular weights by this technique of the order of 65×10^3 for humic acid extracts from chernozem and sod-podzolic soils but they warn that these values might be influenced by the presence of clay minerals in the preparations.

3.5.3 Molecular Shape and Size

Several of the methods used for determining the molecular weight of polymers can also be used to determine their shape and size, particularly if they involve considerations of frictional forces acting on solute macromolecules as they move through solvent media. The magnitude of these frictional forces is related to the shape and sizes of the macromolecules. Ultracentrifuge and viscosity measurements are particularly effective techniques for such studies, especially when they are used in conjunction with one another.

Before becoming involved in more detailed discussions about shapes and sizes of humic substances we must point out that the data which can be obtained in the laboratory do not necessarily provide accurate representations of the shapes and dimensions of these macromolecules in the soil. The laboratory experiments are carried out in solution whereas humic substances in the soil are largely in the solid or gel phase. Thus although the solution shapes and dimensions are important for an understanding of the system *per se* they need not necessarily be accurate representations of those pertaining in the soil. Many of the criticisms made earlier concerning the experimental conditions under which measurements were made in ultracentrifugation studies also apply to data recorded in this section. Thus proposals with regard to molecular configurations have not always been based on sufficiently sound experimental observations.

Information from frictional ratios

Interpretations about molecular shapes are often derived from a consideration of values obtained for the frictional ratio f/f_0, where f is the frictional coefficient of the molecule in question and f_0 the frictional coefficient for a condensed sphere (containing no solvent) occupying the same volume. Thus the value of f/f_0 will be a measure of the deviation of the molecule from a spherical shape and/or condensed form. Tightly coiled globular proteins, as would be expected, give f/f_0 values of 1.0 or only slightly greater. However, the frictional ratios for molecules which are highly solvated or are more extensive in one direction than another often give rise to values of approximately 2 to 3, and for extreme cases, such as the rod-shaped nucleic acids, even higher values are obtained. Flaig and Beutelspacher (1968) suggested that the humic acid molecule adopted a roughly globular conformation based on frictional ratio values of approximately 1.1, but these values were obtained from ultracentrifugation work in which charge effects were not suppressed. When salt was added their results gave the higher frictional ratio values of 1.2 and 1.5. This would indicate that the molecules were considerably distorted to ellipsoidal rather than spherical shapes, and with the

longer axis several times greater than the shorter axis. Piret *et al.* (1960) used a combination of ultracentrifuge and viscosity measurements to obtain frictional ratios of 1.6 to 1.86. Their results indicated to them that the configurations of the molecule approximated to a prolate ellipsoid with axial ratio of about 7:1, and also that the structure was likely to be hydrated rather than condensed. Stevenson *et al.* (1953) reported a still higher frictional ratio indicating an even greater degree of asymmetry and/or hydration.

Once again the determinations of frictional ratio values require the averaging to a single figure of the large variations in the molecular parameters of the sample being measured. As we have already seen from the consideration of molecular-weight values this can result in gross distortion in the interpretation of the data. It is probable that advances in protein chemistry, in which rigid shapes such as globular spheres and oblate or prolate ellipsoids were found to be good approximations of the shapes of the molecules, have had an undue influence on the thinking of workers involved in determining the shapes of humic molecules. Before discussing the validity of such comparisons attention should be given to differences in the origins, chemical compositions, and the structures of the different molecules, as well as to comparisons of properties such as hydration and ion-exchange behaviour. From considerations of the regularity of protein structure, the nature of the bonds and cross-linkages which hold the protein molecules together, and the low hydration of spherical globular proteins, it would be difficult to visualize how humic substances, on the basis of what we know about them, should have similar shapes or tertiary structures. Certainly evidence which suggests similarities of shapes should be subjected to rigorous appraisal.

The ultracentrifuge study of well-separated humic acid fractions of low polydispersities by Cameron *et al.* (1972b) have given some reliable frictional ratio values. These fractions covered a wide range of molecular weight values and they showed that the values for the frictional ratio varied as a function of molecular weight. Now it can be shown (Tanford, 1961) that for shapes other than the hard sphere, represented by some globular proteins, the frictional ratio f/f_0 will vary as a function of molecular weight. It can be further shown that this function will be dependent upon the configuration of the polymer, e.g. spherical, oblate and prolate ellipsoids (all rigid), and flexible, random coils.

Comparisons of the experimental frictional data with the theoretical relationships derived from a consideration of these models indicated that only the oblate ellipsoid (rigid discus shape), and the flexible, random coil need be considered. As discussed above humic materials are unlikely to possess the degree of ordered structure necessary to maintain a rigid configuration. Their mode of formation, heterogeneous nature, and particularly their polyelectrolytic properties in solution are more likely to result in a less rigidly structured molecule whose polar and charged functional groups will be hydrated. Also the dissociation of charges, as discussed already, will lead to intramolecular repulsion causing molecular expansion. These are among the factors which, combined

with additional experimental evidence, caused Cameron *et al.* (1972b) to propose the random coil model for humic acids. This model satisfied the observed experimental behaviour of the humates better than any other, and it is consistent with what might be predicted from considerations of the formation, environment, and chemical and physical behaviour of humic substances.

If it is assumed that this model is correct, or at least provides a good working hypothesis, it is worthwhile considering the properties it predicts and to compare them with experimental observations. A randomly-coiled humic acid molecule in solution can be visualized as a strand, with charges distributed along its length, which coils randomly with respect to both time and space. This results in a molecule which is roughly enclosed by a spherical shape within which the distribution of molecular mass is Gaussian, i.e. the mass density is greatest at the centre and decreases to zero at the outer limits of the sphere. The shape of the Gaussian mass distribution will vary according to whether the molecule is tightly or loosely coiled, and this will in turn depend upon factors such as the extent of solvent penetration, charge density, degree of dissociation, the nature of the counterion etc. Chain branching, which is thought to occur in humic substances, gives rise to a higher mass density within the sphere and results in a molecule that is more compact than a single linear chain of the same molecular weight.

In the study under discussion (Cameron *et al.*, 1972b) chain branching was

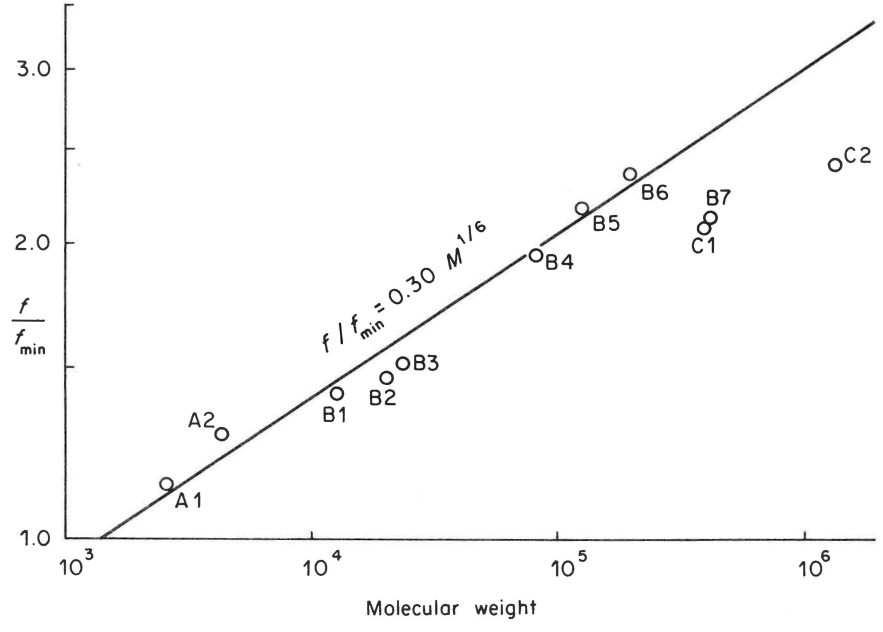


Figure 3.18 Experimental relationship between the frictional ratio and molecular weight of humic acid compared to a theoretical curve for linear random coils under theta solvent conditions. (After Cameron *et al.*, 1972b)

Table 3.5 Molecular parameters of humic acid fractions (A–C) separated by gel chromatography and subjected to ultracentrifugation experiments (Cameron *et al.*, 1972b). Reproduced by permission of Oxford University Press

Fraction	Extractant	Sedimentation[a]		$D_{20} \times 10^7$	$M \times 10^{-3}$	$[f] \times 10^5$	f/f_{min}	$\frac{R_G}{M^{1/2}} \times 10^9$	R_G
		Conc. g dm^{-3}	$s_{20} \times 10^{13}$ sec.	cm^2 sec^{-1}		dyne cm^{-1} sec poise^{-1}		cm	nm
A1	Na pyro-	1.2	0.82	21.4	2.6	0.19	1.14	2.9	1.5
A2	phosphate	2.4	1.02	16.0	4.4	0.25	1.28	3.0	2.0
B1		1.2	1.88	10.2	12.8	0.40	1.41	2.8	3.2
B2		1.2	2.47	8.4	20.4	0.48	1.46	2.7	3.8
B3		1.2	2.64	7.7	23.8	0.52	1.52	2.7	4.2
B4	20 °C	1.2	4.7	3.95	83	1.02	1.96	2.8	8.2
B5	NaOH	1.2	5.6	3.07	127	1.32	2.18	2.9	10.5
B6		1.6	6.7	2.45	199	1.65	2.35	2.9	13.2
		0.8	7.2						
		0.8	7.2						
B7		1.2	12.6	2.13	412	1.90	2.12	2.4	15.3
C1	60 °C	1.2	12.6	2.15	408	1.88	2.11	2.4	15.0
C2	NaOH	2.4	24.6	1.26	1360	3.21	2.41	2.2	25.5

[a] Sedimentation coefficients obtained by a least squares linear fit had typical standard errors of 4 per cent for lower molecular weight fractions, decreasing to 2 per cent at higher molecular weights.

Mean diffusion coefficients, obtained from 4 to 8 replicate runs, had standard errors between 1 to 5 per cent. Solution concentrations were approximately 0.1 g dm^{-3}.

thought to be particularly prevalent at high molecular weight values and the resulting more compact molecular dimensions caused f/f_0 to be lower than the theoretical prediction (Figure 3.18). The molecular coil in solution is perfused with solvent which can exchange with more solvent outside the sphere of influence of the macromolecule. However, because of the higher density of polymer chains in the central regions the rate of solvent exchange is relatively slow, and in the more compact structures the solvent is essentially trapped. This trapping effect is relatively small for the low molecular weight polymers but it increases markedly at higher molecular weights. Thus for the denser structures we can consider that the solvent is able to flow freely through the periphery of the molecule but that this freedom of movement is gradually restricted as the centre of the molecule is approached, and that finally the solvent is effectively trapped in the central regions. Thus the shape of the molecule in solution is essentially spheroidal but not condensed or rigid.

The values obtained for the spherical radii of the molecules are shown in Table 3.5 and range from 1.5 nm for a molecular weight of 2500 to 25.5 nm for a molecular weight of 1.4×10^6. These values agree well with some of those reported by Flaig and Beutelspacher (1968). Their data were also obtained from ultracentrifugation experiments in which charge repulsion effects were repressed, and they derived a radius figure of 6.9 nm for a molecular weight of 77×10^3 (*cf.* Table 3.5). When charge effects were not suppressed much lower values were obtained.

Other techniques have not given such well defined data. For instance Flaig and Beutelspacher (1951) report diameters of up to 10.0 nm using electron microscopy. This value is somewhat smaller than the maximum figures obtained from ultracentrifugation but perhaps not unreasonable because of the shrinking which must take place when the sample is dried for the microscopy. Wershaw *et al.* (1967) report maximum radii of 11.0 nm measured by X-ray scattering for sodium humate in solution. Their frictional coefficients were found to be between 1.3 and 2.5 showing that the molecules were not condensed and exhibited a degree of deviation from a true sphere. This technique would appear to be very promising, and although some fractionation of the samples used was achieved by gel chromatography it is probable that significant refinements in the data could be achieved by using well fractionated samples.

Influence of charge on the shape of humic substances

As we have seen from our earlier considerations of the properties of humic substances these materials contain considerable amounts of acidic and other functional groups which give rise to many charged sites in the polymer. Such sites will greatly affect the properties and behaviour of the molecules, both in solution and in the solid state, and we will now consider what some of the effects might be.

If, for the sake of argument only, we were to consider the molecular configuration of humic substances to be that of condensed, rigid structures rather than more open random coils, and further that this configuration is retained in solution then we would have to take into account a number of criteria. It would be especially necessary that all of the charged sites which are dissociated be located on or near the exterior of the molecules. Charge sites within the matrix of the polymer could not dissociate for two reasons; firstly, solvent which is required for dissociation is absent from the inner parts of a condensed structure, and secondly if dissociation of internal charged sites did occur then the resultant charge repulsion would lead to molecular expansion to destroy the condensed configuration.

None of these requirements are observed in humic substances. For instance they manifestly lack the ordered structure which would be necessary to ensure that all of the numerous charged sites are located at the surface. Indeed, all available evidence indicates that the charged sites are distributed along the length of the polymer and thus they can occur at any point in the polymer matrix. Observations of swelling properties when solvated, and of ion-exchange behaviour when in solution show that the molecules are readily penetrated by solvent and that most charged sites are dissociated and accessible for ion exchange. Such properties are incompatible with a condensed, rigid molecule but are compatible with the proposed flexible, random-coil model which is capable of molecular expansion.

3.5.4 The Solid or Gel Phase

As already emphasized (Section 3.5.3), the experimental determination of molecular shape is carried out in solution and the resulting description applies strictly only to the molecule when it is in this state. However, we can use the solution to predict what might occur when the molecule is precipitated by the addition of suitable counterions, or is dehydrated. In this way we can get some idea of what the molecular configuration might be when the humic substances are in their more common form in the soil i.e. in the solid or gel state. In order to do this it is probably more informative to consider first the forces and effects which cause a polymer such as humic acid to dissolve and remain in solution. Firstly, there are solvation effects which have been discussed in Section 3.2.1. Under normal aqueous conditions the solvent is water which will perfuse the molecular structure generally, but will solvate certain portions more strongly. For instance the polar functional groups, usually containing oxygen, but some with nitrogen or sulphur, and the charged sites (Section 3.2.2) plus their counterions will be strongly hydrated. However, solvation processes alone are not sufficient to carry any except the lowest molecular weight humic substances into solution, and reference has been made (Section 3.2.2) to the necessity for a degree of charge dissociation which allows some counterions to move into bulk solution and gives rise to electrostatic repulsion by the residual charges in the polymer, and eventually gives rise to the solution of the macromolecule. Although it is obvious that the process of molecular expansion due to the mutual repulsion of dissociated charge sites will have a very profound effect, there is a limit to the extent to which this expansion occurs. Thus, even when dissociation is complete a large proportion of the counterions, although separated from their charged site, are still found in the solvent enclosed by the polymer coils. These therefore remain within the domain of the macromolecule which resists further expansion. The foregoing considerations apply when the counterions are readily dissociated cations, such as sodium, potassium, and ammonium.

The situation is different when H^+, Ca^{2+}, and polyvalent cations such as Fe^{3+} are the counterions and these give rise to precipitation of the humic substances. These ions are either undissociated or only very weakly dissociated in the presence of humic substances and thus one of the major solubilizing forces is lost. When the polymer is H^+-saturated hydrogen bonding between the molecules shrinks the structure. While the introduction of counterions such as H^+, Ca^{2+}, and Fe^{3+} will not affect the solvation of functional groups or charged sites it will greatly decrease the amount of solvent within the polymer matrix. Thus the absence of charge repulsion will lead to molecular shrinkage and in the case of multivalent cations the simultaneous linking across two or more charged sites will cause further molecular contraction. Hence, in the processes of charge neutralization with non-dissociating ions, solvent expulsion and molecular shrinkage occur simultaneously, and result in precipitation. Thus, molecules of

humic substances in the gel or solid state will be more compact, more tightly coiled and much less solvated compared with those in the solution state.

Even so, the molecule will still be far removed from a condensed form since there will still be a considerable degree of solvation of functional groups and charged sites. Further shrinkage and condensation can be induced by removal of water through drying, and under such conditions the molecules may well move into a highly condensed state. In this condition strong intramolecular bonds will be set up particularly between hydrophobic portions of the molecule. Such interactions probably account for the observed difficulty in rewetting organic soils and humic materials which have been exhaustively dried.

3.5.5 Investigations of Polyelectrolyte Properties of Humic Substances

We have repeatedly referred to the polyelectrolyte nature of humic materials because they exhibit many of the properties commonly associated with charged macromolecules, including polybasicity and ion exchange. Several techniques have been developed for examining the polyelectrolyte properties of biopolymers in particular, and many of these can be applied to investigations of humic substances.

Electrophoresis

This is one of the procedures used to describe the movement of ions in an electric field. When a particle of charge q is placed in an electric field of strength E it will be subject to a force Eq and will achieve a steady-state velocity μ under this force, where

$$\mu = Eq/f \tag{3.54}$$

and f is the frictional coefficient. For a charged macromolecule of radius R and carrying Z unit charges,

$$q = Z\varepsilon \tag{3.55}$$

where ε is a unit charge, i.e. 4.3×10^{-10} e.s.u. The frictional coefficient is given by

$$f = 6\pi\eta R \tag{3.56}$$

where η is the viscosity of the medium through which the particle is travelling and R is the radius of the particle. Thus the steady-state velocity of the molecule becomes

$$\mu = \frac{EZ\varepsilon}{6\pi\eta R} \tag{3.57}$$

where μ is the electrophoretic velocity. It is more usual, however, to determine a parameter which is not dependent on the strength of the electric field but only

on the parameters of the charged molecule. This is the electrophoretic mobility U, defined as

$$U = \mu/E \tag{3.58}$$

Thus the technique would appear to offer an easy method for determining and comparing molecular sizes and charge densities.

Whilst this theoretical approach is straightforward the actual study of polyelectrolytes by electrophoresis is complicated by a number of factors. Macromolecular ions do not exist in isolation in solution but are accompanied by sufficient counterions to give an electrically neutral system. These counterions will not be randomly distributed through the solution but will tend to form localized clouds around the macromolecules. Under the influence of the electric field the counterions will tend to move in the opposite direction to the macroions, and in addition they will carry relatively large amounts of solvent with them. Both of these effects exert a drag on the movement of the macromolecules to give a resultant electrophoretic mobility below that predicted, and by an amount that is difficult to calculate. Another complication arises from the variable pK_a values which are exhibited for the acidic functional groups of polyelectrolytes in general and for humic substances in particular. At any given pH value it is difficult to estimate correctly the number of dissociated sites. Even if all sites are dissociated not all of the counterions will be outside the polymer matrix and so it will be very difficult to estimate the charge on the macromolecule. In any case it is considered that the charge or potential at the surface of the molecule should be replaced by the zeta-potential, which is the potential at the surface of shear of the charged particle. This relates to the true surface of the moving particle and includes solvation layers and attendant counterions. The zeta-potential is then somewhat lower than the potential at the surface of the molecule. In practice, various salts or buffers are added to the system to suppress deviations from ideality and to create and maintain an electrically neutral, pH-buffered system. Since added positive and negative ions will usually have different electrophoretic velocities the theoretical problem of estimating the final electrophoretic mobility of the macro-ion is further complicated and very difficult to determine.

It is obvious from this discussion that electrophoresis can be used to give semi-quantitative information about molecular parameters such as charge, size and shape, or to provide a fractionation based on molecular variations related to these properties. However, it is only of limited value for giving accurate measurements of actual molecular parameters, and its use for fractionations has been superceded by gel chromatography used in conjunction with ion-exchange chromatography techniques. Various types of electrophoresis procedures have been used to fractionate humic substances, and these are reviewed by Flaig *et al.* (1975).

Paper electrophoresis in alkaline buffers is the most common form of the technique used to fractionate humic materials. Other support media such as

columns of glass or gel beads have also been used. If, however, the electrophoretic mobility is to be measured then it is best to employ the free boundary technique (Stevenson *et al.*, 1952). As with other fractionation procedures discrete fractions are not obtained but rather there is a gradation of properties with one fraction merging into another. In general, however, gray humic acids migrate very slowly, brown humic acids migrate more quickly and fulvic acids migrate more rapidly still. This observed electrophoretic behaviour supports the evidence and the view that the observed sequence is composed of molecules of increasing charge densities and decreasing molecular weights.

Many workers have observed fluorescent areas or fractions during electrophoresis experiments (e.g. Waldron and Mortensen, 1961), and this behaviour is usually associated with the more mobile materials. It is not clear whether the fluorescence is due to separate components or is characteristic of particular fractions of humic substances. It is possible that all fractions do in fact fluoresce, but that this is masked by the intense absorption of the grey-brown components.

Electrofocusing

This technique is similar to electrophoresis, and was designed to separate protein mixtures by utilizing their different isoelectric points. A column of gel is prepared having a pH gradient from top to bottom, and the gel material is instrumental in stabilizing the pH gradient. The mixture is applied to the top of the column and a potential difference is then applied between the top and the bottom. Under the influence of this field the charged molecules move until they reach a point in the column where the pH corresponds to their isoelectric point. At this point there is no net charge on the molecule and it ceases to move despite the electric field.

Although humic substances do not have an isoelectric point they can be fractionated by electrofocusing procedures (e.g. Cacco *et al.*, 1974). When there is an appropriate range of pH values within the gradient the humic substances will move until a sufficiently acidic pH value is reached where all the negatively charged sites are protonated and thus the molecule will cease to move because they are not dissociated. In this sense the process is more analogous to fractional precipitation than to isoelectric focusing, but nevertheless it does provide a potentially useful fractionation technique. Electrofocusing procedures provide a useful method for characterizing humic extracts from different soil types.

Ion exchange

One of the most important properties of soil humic substances is their ability to act as ion exchangers. This capacity to retain and exchange cations plays an important role in the supplying of nutrients to plants and for the general maintenance of soil fertility. The exchange capacity of humic materials is very high compared with most soil mineral components, and although they are generally

less abundant than the inorganic colloids they often make a major contribution to the overall cation-exchange capacity of the soil. Humic materials are also highly efficient at combining strongly with a large number of heavy metal cations, some of which are essential plant micronutrients. Thus the behaviour of humic materials as an ion exchanger and complexing agent is of prime importance in the retention, mobilization, and cycling of nutrients.

Ion-exchange processes and the complexing of heavy metal ions are dealt with more extensively in the companion volume, and only aspects which relate to the chemical or molecular structure of humic substances will be considered here. As discussed earlier (Section 3.5.2) the molecular structure will profoundly influence accessibility to the ion-exchange sites and also the kinetics and mechanisms of exchange.

The first effect to consider is the Donnan membrane effect, the theory of which is dealt with in more detail in Chapter 5. Consider a solution containing macro-ions and counterions separated by a membrane from a solution containing only simple small ions. The small ions can pass through the membrane but the macro-ions cannot. When equilibrium is reached the distribution of diffusible, small ions is not uniform through the system. Counterions, which have charges opposite in sign to those on the macro-ions, will be present at higher concentration on the macro-ion side of the membrane and co-ions, which carry the same charge sign as the macro-ion, will be found in higher concentration on the other side of the membrane.

Whilst this theory has been derived and shown to be valid for the system described it has also been found to apply to single, charged, colloidal particles or macromolecules. In this case, instead of an actual membrane an imaginary boundary can be drawn around the particle or molecule (usually at a short distance from the particular or molecular surface) encompassing a small amount of solvent. Solvent and small ions can pass freely across this imaginary boundary, but the macro-ion obviously cannot, and the boundary thus becomes in effect analogous to a semipermeable membrane. The importance of this, as far as polyelectrolyte molecules are concerned, is that counterions can pass freely through the imaginary boundary and perfuse through the matrix of the molecule, whereas co-ions carrying the same charge as the macro-ion will be kept at a low concentration within the boundary and will be effectively excluded from the volume contained within the imaginary boundary. This phenomenon is often referred to as ion-exclusion, and the effect can be very large, particularly at low electrolyte concentrations, but it can, nonetheless be reduced by the addition of the electrolyte.

Since co-ions are excluded from the macro-ion the mechanism of ion exchange is now more apparent. Counterions from the solution enter the matrix of the exchanger and, in order to retain electrical neutrality, an equivalent number of other counterions must be displaced and move out of the matrix. Ion exchange is thus a simple stoichiometric process. The actual process of replacing one ion by another at an exchange site is very rapid and the rate at which ion-exchange

processes occur is determined by a slower process, namely the rate at which ions diffuse to or away from the exchange sites. Since two different types of ions are usually involved this process is often referred to as interdiffusion.

In practice two separate, potentially rate determining processes can be identified. The first concerns the movement of ions within the matrix of the exchange medium and is referred to as *particle diffusion.* This process can become the rate limiting step when movement of ions through the particle to the exchange sites is limited. Such conditions can occur if the polymer is densely coiled and cross-linked. This leads to large frictional forces and tortuous pathways, and is accentuated if the diffusing ions are large and/or exhibit specific interactions with the polymer matrix. The second process concerns the rate of diffusion of ions across the adherent film of strongly held solvent adjacent to the polymer surface. The interdiffusion of ions across this zone is referred to as *film diffusion.* If the rate of particle diffusion is high and exchange within the particle is fast then film diffusion becomes the rate controlling step.

It has been shown (Helferich, 1962) that the nature of the rate controlling step can be represented mathematically using the expression

$$\frac{X\bar{D}S}{CDr_0}(5+2\alpha_B^A) \equiv 1 \tag{3.59}$$

where X is the concentration of fixed ionic sites, C the concentration of the counterion in solution, $\bar{D}$ the interdiffusion coefficient in the ion exchanger, D the interdiffusion coefficient in the surrounding film, S the film thickness, r_0 the radius of exchanger, and α_B^A is a separation factor or selectivity coefficient. When this expression is equal to one, the half-times for film and particle diffusion are equal; if it is much greater than one then diffusion within the particle is the rate limiting step, and if it much less than one then diffusion through the surrounding film of liquid is the rate limiting step.

There is only limited awareness about the mechanisms and kinetics of the ion-exchange process in humic substances although much is known about the relative binding strength of the polymer for a wide variety of metal and other cations. The evidence which is available indicates that particle diffusion is the rate controlling step. However, the attainment of equilibrium for humic acid is found to be comparable with moderately cross-linked ion-exchange resins but more rapid than for highly cross-linked resins. In addition exchange appears to be complete when equilibrium is reached. These observations indicate that the humic substances have a structure sufficiently complex to cause some restriction to the rate at which exchange occurs, but that the structure is sufficiently open to allow access to all of the exchange sites. This concurs once more with the earlier structure, proposed in Section 3.5.3, of a moderately cross-linked random coil, but it is not compatible with any of the condensed structures which have been proposed.

Whilst structural organic cross-linkages are not thought to be numerous,

other bridging linkages can be formed when the negative charge of the molecule is balanced by di- and trivalent cations. Thus when salts such as calcium-, iron-, or aluminium-humates are formed the effects produced are somewhat analogous to cross-linking, and molecular shrinking is observed. In this situation tortuosity and general frictional forces within the molecule will be greatly increased and the passage of solvent and of ions through the macromolecule will be restricted. As a result the ion-exchange process may well be slowed down and equilibrium achieved more slowly. Processes such as these, involving the more strongly held cations, are of great importance in the soil, but there is little evidence about the way in which they effect ion-exchange processes. There is considerable scope for more detailed studies on the ion-exchange processes which take place in humic substances, particularly in the area where these studies relate more closely to the actual conditions and exchange processes which occur in the soil.

3.6 SOIL POLYSACCHARIDES

Soil polysaccharides are composed of hexose (six-carbon), and pentose (five-carbon) sugar units and of some hexose derivatives. On average they make up about 10 per cent of the soil organic matter. Interest in these colloidal materials began during the mid 1940s when Martin (1945, 1946) established that slimy bacterial products, earlier shown (Waksman and Martin, 1939) to aggregate sand–clay mixtures, were polysaccharides. At about the same time (Haworth *et al.*, 1946) and Geoghegan and Brian (1946, 1948) were investigating the uses of bacterial dextran and levan polysaccharide slimes for the improvement of soil structure. Since then sporadic research interest has been directed to investigations of the genesis, isolation, purification, structural determinations, and the roles of polysaccharides in the soil environment.

During the 1950s statistical analysis (e.g. Rennie *et al.*, 1954; Chesters *et al.*, 1957) and in the early 1960s chemical treatments such as periodate degradations (e.g. Greenland *et al.*, 1961, 1962; Clapp and Emerson, 1965) further established the role of polysaccharides in the aggregation of soil particles. In the meantime Mehta *et al.* (1960) had shaken confidence in the aggregating influences of soil polysaccharides by showing that the crumb-structure of a Swiss braunerde resisted degradation in a periodate medium, but the later work by Greenland *et al.* (1961) suggested that these resistant crumbs of high organic matter contents were held together by fungal hyphae and myceliae, and other non-polysaccharide materials.

There are a number of reviews which deal with various aspects of the isolation, compositions, and the roles in the soil of sugars and polysaccharides (Mehta *et al.*, 1961; Gupta, 1967; Swincer *et al.*, 1969; Finch *et al.*, 1971; Greenland and Oades, 1975). Here we will deal briefly with the isolation, fractionation and purification, composition, and in a general way with structural determinations. Some consideration will also be given to polysaccharide clay interactions since these are likely to be important in soil aggregation processes.

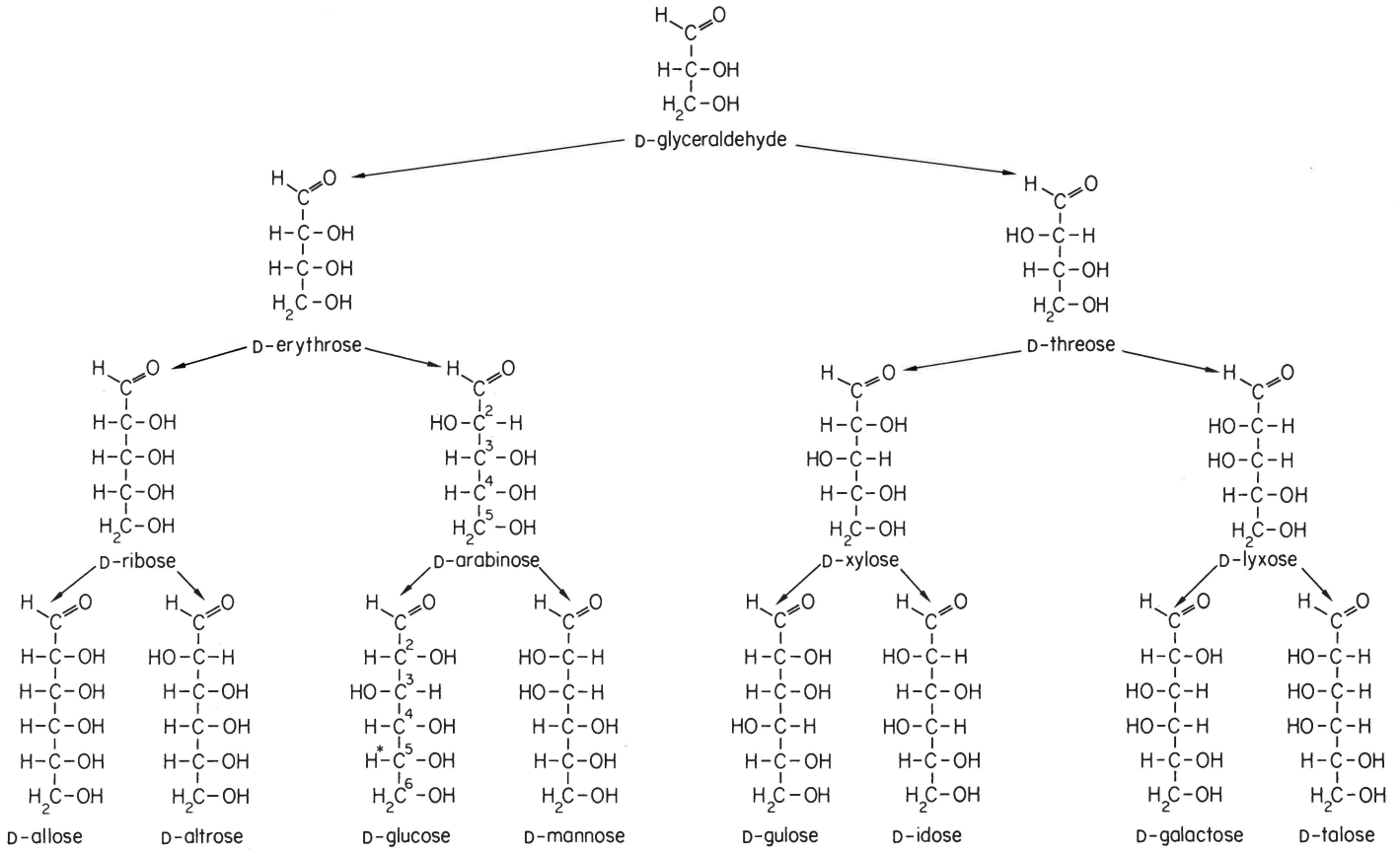


Figure 3.19 Open chain structures of aldoses in the D-configuration

3.6.1 The Composition and Structures of Monosaccharides

Before the composition, possible structures and properties of polysaccharides can be appreciated it is necessary to have an understanding of the structures of the sugar units which compose them. This treatment will provide an elementary knowledge of sugar structures. Texts on the chemistry of saccharides, such as those by Coffey (1967), Davidson (1967), Guthrie and Honeyman (1968), and Pigman and Horton (1970) give detailed treatises on the compositions and structures of monosaccharides (single sugar units), oligosaccharides (containing up to ten sugar units linked together), and polysaccharides. We will be concerned only with aldoses (which can contain an aldehyde functional group) although ketoses, especially fructose, have been found in soils.

It is easy to derive the aldopentose and aldohexose structures by starting from glyceraldehyde. All of the sugars listed in Figure 3.19 have the D-configuration which refers to the highest numbered asymmetric carbon atom (e.g. carbon-4 in D-arabinose, carbon-5 in D-glucose). The sugars are arranged systematically. By following the arrows it can be seen that two daughter sugars are built from each parent molecule, and the hydroxyls on the carbons α- to the aldehyde groups are first placed on the right hand side of that carbon and then on the left hand side. When all of the structures are drawn the names of the pentose and hexose structures can conveniently be remembered by the use of mnemonics. Thus *RAXL* refers to ribose, arabinose, xylose, and lyxose and the favoured mnemonic for the hexoses '*all altruists gladly make gum in gallon tanks*', has some resemblance to the names *allose*, *altrose*, *glucose*, *mannose*, *gulose*, *idose*, *galactose*, and *talose*. Note also that the arrangement of the hydroxyl groups follow a pattern. Thus in all cases the hydroxyl on carbon number 5 (C-5) are on the right, the first four of C-4 are on the right, and the second four on the left and so on. The L-sugars, which are the mirror images of the D-structures are less common in nature.

A solution equilibrium exists between the open chain structures and cyclic forms of pentose and hexose sugars. However, more than 90 % are in the cyclic form at any one time. Furanose (five-membered) and pyranose (six-membered) structures result from cyclization, through the formation of hemiacetal functional groups between C-4 and C-1 and between C-5 and C-1, respectively, as illustrated for the arabinose and glucose structures in Figure 3.20. Cyclization generates a new asymmetric centre at C-1 and thus gives rise to α- and β-anomers of the D- and L-sugars. These anomers are shown for the α-D- and for β-D-arabinose structures in Figure 3.20.

Five-carbon ring structures are almost planar whereas the cyclic six-carbon structures are puckered and assume conformations, or positions in space ranging from the '*boat*' to the '*chair*' extremes. The presence of one heterocyclic oxygen in the ring does not appreciably alter the conformations. Six-membered cyclic structures will preferentially assume the chair conformation where non-bonded interactions involving the bulky substituents (—OH and $—CH_2OH$

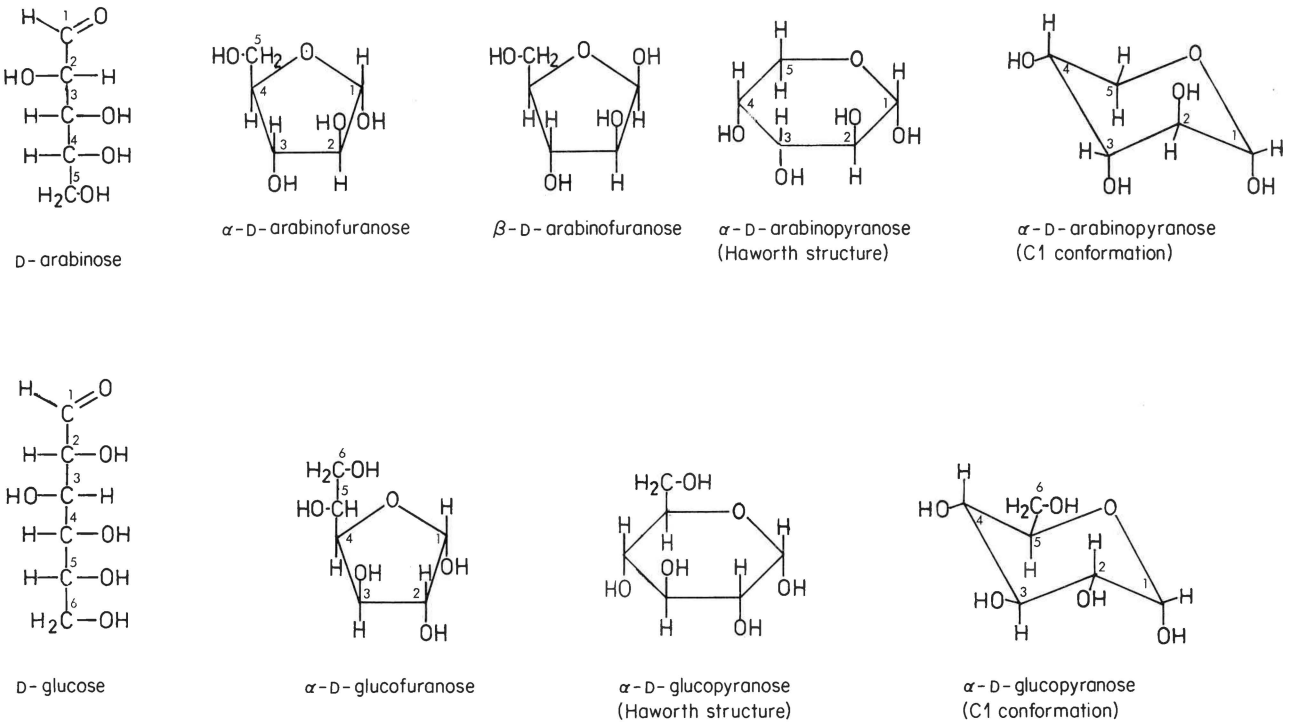
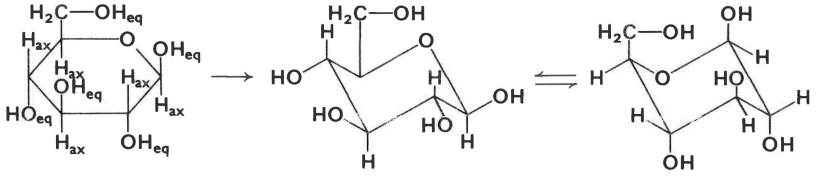


Figure 3.20 Open chain and ring structures of D-arabinose and D-glucose

groups in Figure 3.20) on the carbon atoms are at a minimum. However, these six-membered structures are still often represented by Haworth formulas as shown. From examination of the structures in Figure 3.20 it will be seen that assignment of the hydroxyl groups from the open-chains to furanose and Haworth structures is straightforward. It is also easy to convert the Haworth formulas to the C1 chair conformations shown. The sequence of transformations for β-D-glucopyranose, shown in scheme (3.60), illustrates how substituents or carbons of Haworth structures are assigned in the C1 conformations. The 1C conformation is obtained by 'flipping' the model for the C1.



(3.60)

Haworth structure β-D-Glucopyranose (C1) β-D-Glucopyranose (1C)

The C1 and 1C conformations are interconvertible in solution. However, the preferred and most stable conformation will be that in which the non-bonded interactions are least, and this is achieved when the bulky substituents are all equatorial as seen for the β-D-glucopyranose (C1) structure. By use of atomic models it is easy to realize how non-bonded interactions are greatest when the bulky groups are axial, in positions 1, 3, 5 and in 2, 4, because these two groups of positions are on the same side of the cyclic structure. Stacey and Barker (1960) have suggested that the favourable axial–equatorial arrangements of the bulky substituents could partially explain the abundance of D-glucose, D-galactose, and D-mannose in nature. This does not, however, explain the absence of D-allose and D-lyxose from polysaccharide structures.

Six derivatives of hexose sugars are found in soil polysaccharides. These are fucose, or 6-deoxy-L-galactose, rhamnose, or 6-deoxy-L-mannose (in which the $—CH_2OH$ groups on C-6 are replaced by $—CH_3$), glucosamine, or 2-amino-2-deoxy-D-glucose, galactosamine, or 2-amino-2-deoxy-D-galactose (in which the —OH groups on C-2 are replaced by $—NH_2$), glucuronic and galacturonic acids (in which the $—CH_2OH$ groups on C-6 are replaced by $—CO_2H$). The amino sugars are likely to be present in the polysaccharides as the acetyl ($—NHCOCH_3$) derivatives, but the acetyl group is lost during hydrolysis.

3.6.2 Structures of Polysaccharides

Polysaccharides are polymers in which acetal linkages are formed by elimination of water through condensation of the anomeric or glycosidic hydroxyl (on C-1) of one sugar with any non-glycosidic hydroxyl of another sugar molecule. The sugar residues released by the hydrolysis of a polysaccharide compose its

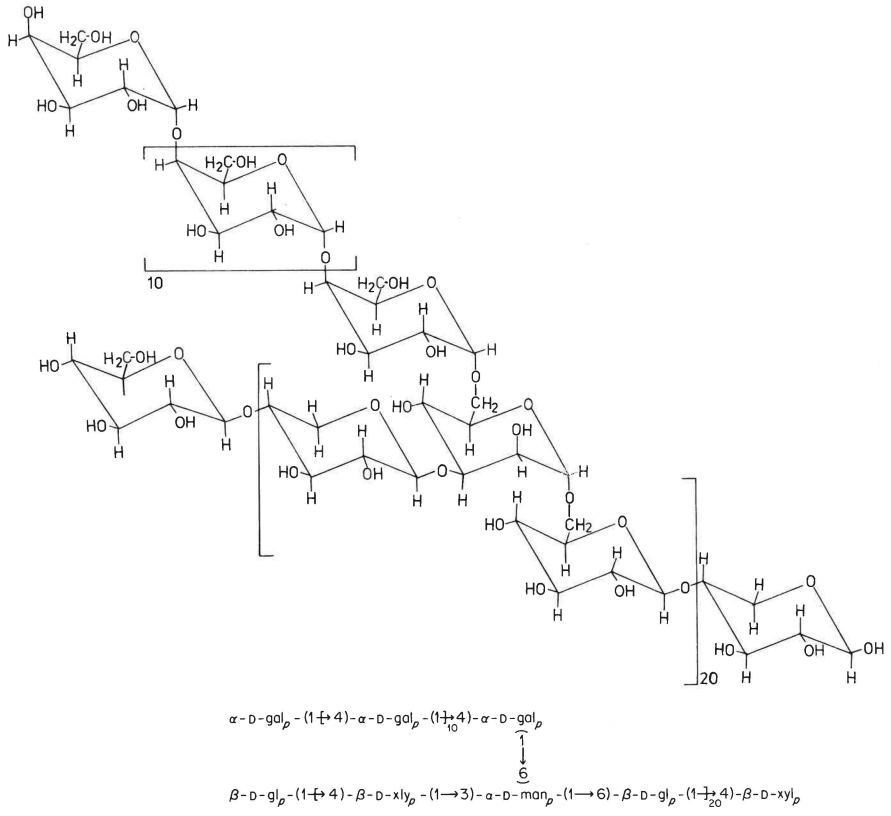


Figure 3.21 Diagrammatic representation of the structure of a branched heteropolysaccharide

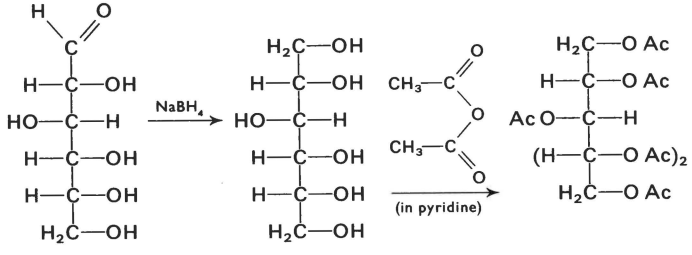
primary structure, and the secondary structure is determined by the sequence of these sugars in the polymer chain. Figure 3.21 represents the secondary structure of a hypothetical polysaccharide, whose primary structures are composed of β-D-glucopyranose, β-D-xylopyranose, α-D-mannopyranose and α-D-galactopyranose units. A recognized notation which describes the cyclic monosaccharide components (and where *p* refers to the pyranose structure), and the types of linkages are also given. The repeating groups are enclosed by the large square brackets and the numbers 20 and 10 indicate that these groups are repeated 20 and 10 times in the 'backbone' and side chain, or branch, respectively.

Polysaccharides, sometimes referred to as glycans, can be broadly classified as homopolysaccharides (composed of one repeating monosaccharide) and heteropolysaccharides (composed of two or more monosaccharides). These structures are further subdivided into straight-chain and branched structures, and the polysaccharide in Figure 3.21 can be classed as a branched heteropolysaccharide. In this case the branching takes place through the α-D-galactopyranose(1 → 6)-α-D-mannopyranose linkage.

Bouveng and Lindberg (1961), Whistler (1965), Björndal *et al.* (1973) and Aspinall (1976) give details of the principles and techniques for determining polysaccharide structures. We present here only the rudiments of some procedures which can be used for structural determinations.

In order to ascribe detailed structure to any polysaccharide it is necessary: (a) to know what monosaccharides compose it; (b) to determine the relative abundances of these sugars; (c) to establish which hydroxyl groups are involved in the linkages in each case, and whether the glycosidic linkages are α or β; (d) to know the average chain lengths of the 'backbone' and of the branches; and (e) to know the extent of branching and the branching positions.

Determination of primary structure. Hydrolysis in acidic media is generally used to release the monosaccharide components from polysaccharides. This technique and the mechanisms involved have been reviewed by BeMiller (1967). It must be realized that losses occur during acid hydrolysis. These occur most frequently through the formation of furfuraldehyde derivatives (from the pentose and hexose residues released) which form brown polymeric structures. The extent of polymer formation can be decreased by carrying out the hydrolysis *in vacuo* or in an atmosphere of nitrogen. The hexopyranoside (glucose, galactose and mannose) linkages in the polysaccharide are more resistant to hydrolysis than the xylopyranose structure and advantage can be taken of this in order to achieve a partial hydrolysis.



(3.61)

The sugars released by hydrolysis can be identified by paper chromatography and quantitatively determined by eluting the spots followed by colorimetric determination. More accurate identifications and determinations use gas–liquid chromatographic separations of the alditol acetate derivatives, formed from the alditol (or sugar alcohols) derivatives, as outlined for glucose in reaction scheme (3.61). Earlier we stated that less than 1 % of glucose is in the open chain form. However, as the glucitol is formed by reduction more of the straight chain structure becomes available through the equilibrium process until the reaction reaches completion. Quantitative determinations of the sugars, or of their derivatives, not only provide the primary structure of the polysaccharide but also give the ratios of the different sugar units present.

Determination of secondary structure. When polysaccharides are methylated, methyl ether (—OCH_3) derivatives are formed from all of the free hydroxyl groups. Older methylation procedures used dimethylsuphate in basic solution, or Purdie methylation which employs iodomethane and silver oxide catalyst. More recently procedures have been developed which require only milligram quantities of substrate. For instance, Björndal and Lindberg (1969) successfully methylated 5 mg of polysaccharide, in solution in methylsulfoxide containing methyl sulphinyl cation in an atmosphere of nitrogen, by adding iodomethane. Then the solution was hydrolysed in 0.25M H_2SO_4 and after neutralization the alditol acetates were formed and identified by g.l.c. and m.s. techniques. (See also Lindberg *et al.*, 1973; Dutton, 1974.)

The ether linkages resist hydrolysis in dilute acid, but the glycosidic linkages are, of course, labile. In the case of the polysaccharide structure illustrated in Figure 3.21 the following methylated sugars would be released in the ratios enclosed by brackets: 2, 3, 4, 6-tetra-*O*-methylglucose, (1); 2,3-di-*O*-methyl-xylose (21); 2,4-di-*O*-methylmannose, (20); 2,3,4-tri-*O*-methylglucose, (20); 2,3,4,6-tetra-*O*-methylgalactose, (1); 2,3,6-tri-*O*-methylgalactose (11). In the hydrolysis process both anomers are formed and it is thus not possible to predict from such data whether the residues were involved in α- or in β-linkages. Clearly the 2,3,4,6-tetra-*O*-methylglucose and -galactose can be assigned to non-reducing end groups (i.e. having the glycosidic hydroxyls involved in linkages). The amounts of 2,3,4-tri-*O*-methylglucose shows that this structure must have been derived from glucose in the chain and that the 6-hydroxyl, in addition to the glycosidic hydroxyl was involved in linkages. The same reasoning indicates that mannose was present in the chain and involved in branching through the 6-hydroxyl. The amounts of 2,3,6-tri-*O*-methylgalactose is consistent with a structure in which this sugar composed the branch and was thus involved in the linkage to the mannopyranose in the structural 'backbone'.

The fact that xylose is present in the 'backbone' structure and linked through its 1- and 4-OH groups is indicated by the amounts of 2,3-di-*O*-methyl xylose isolated; but xylose also composes the reducing-end group. The ring structure of the reducing end can open to free the aldehyde functional group, and this can form a phenylhydrazone derivative, or be reduced by sodium borohydride. Such derivatives can be isolated when the polymer is hydrolized to provide proof of the terminal residue containing the free glycosidic structure.

Two problems still remain to be solved: (1) the assignment of the sequence of glucopyranose, xylopyranose, and mannopyranose structures in the backbone, and (2) the determination of the configurations (α or β) of the glycosidic hydroxyls involved in the linkages. By means of partial hydrolysis, using decreasing concentrations of acid, it will be found that the disaccharide composed of the methylated xylose and mannose residues will be present in the greatest abundances as the concentration of acid in the hydrolytic medium is decreased, and the sequence can then be deduced. Enzymatic hydrolysis (Marshall, 1974; Snaith and Levvy, 1973) provides a method for establishing the configurations of

the glycosidic linkages. This requires the addition of specific enzymes in order to cleave the appropriate α- or β-linkage.

Periodate oxidation is another widely used technique in polysaccharide structural studies. However, although meaningful methylation studies can be carried out on purified soil polysaccharides, we do not think that materials of sufficient purity are yet available to carry out periodate studies. Goldstein *et al.* (1965) and the standard texts referenced already give details of the technique.

3.6.3 Origins, Isolation, Purification, and Compositions of Soil Polysaccharides

Finch *et al.* (1971) and Greenland and Oades (1975) have summarized features of saccharides and polysaccharides of plants, animals, insects, and microorganisms, all of which, in one way or another, find their way to the soil environment. There they are subjected to microbial attack and a variety of new polysaccharides are biosynthesized. Cheshire (1977) has summarized studies which have shown that soil microorganisms can synthesize from ^{14}C-labelled glucose (Chahal, 1968; Cheshire *et al.*, 1969; Oades, 1974) or alfalfa (Keefer and Mortensen, 1963) the neutral sugars which are found in soil polysaccharides. However, Cheshire *et al.* (1969) showed that the polysaccharide material formed in this way was deficient in arabinose and xylose compared with natural soil polysaccharides, although the relative proportions of glucose, galactose, mannose, rhamnose and fucose were quite similar.

Cheshire and his associates (see Cheshire, 1977, and references therein) have done much to help our understanding of the origins of soil polysaccharides. Their long-term incubation studies, using ^{14}C-labelled preparations of glucose, glucose plus acetate, xylose, hemicellulose material, and cereal rye straw, indicated that:

1. part of the carbon chain of glucose is incorporated intact in the hexose and deohyhexose residues of newly biosynthesized polysaccharides;
2. the pattern of labelling of the newly synthesized sugars from a xylose substrate is similar to that from glucose, suggesting the possible conversion of xylose to glucose during the biosynthesis processes;
3. at low temperatures, in conditions which depress bacterial but not yeast numbers, the xylose content of polysaccharide from the labelled glucose substrate was similar to that of galactose and mannose, and was presumed to result from synthesis by the yeasts because a high proportion of yeast isolates synthesized xylose;
4. no synthesis from the labelled substrates or arabinose, comparable with that of xylose or the hexoses, was achieved, even when the conditions of light and temperature favoured algal growth (the carbohydrate of algae, like that of plants, contains xylose and arabinose);
5. the xylose in hemicellulose was almost completely degraded after 16 months incubation with soil, and the amounts of labelled glucose and

xylose which remained were influenced by the fineness of grinding of the rye straw.

Much work still remains to be done before the origins of soil polysaccharides can be assigned with confidence. At the present time there is no doubt that many of these polysaccharides, and in particular those containing hexoses and deoxyhexoses, have microbial origins, but the possibility remains that some are plant derived. The arabinose-containing structures, and some of those with xylose, might predominantly come from plants.

Isolation and purification of soil polysaccharides

Before the efficiency of any extractant for soil polysaccharides can be determined it is necessary to know the carbohydrate content of the soil. This can be done by estimating the amounts of sugars in a soil hydrolysate. Of the various hydrolytic procedures, those used by Cheshire and Mundie (1966) and Oades (1967a), based on the studies of Ivarson and Sowden (1962) and Gupta and Sowden (1965), appear to give the best results. The procedure involves pretreatment of the sample with 72% H_2SO_4 followed by dilution to 1N acid and refluxing for a prescribed period. It is advisable to work out the optimum pretreatment and hydrolysis times, because these can vary from sample to sample. Where colorimetric procedures are used (e.g. anthrone), it is desirable to decolourize the hydrolysate products. Accurate quantitative analysis of the different sugars present can be made by the g.l.c. separation (Oades, 1976b) as outlined in Section 3.6.1. However, hexuronic acids (where C-6 is a carboxyl group) are degraded by the hydrolysis process and these should be separately determined (e.g. by the method of Bitter and Muir, 1962).

Methods for the extraction of polysaccharides from soil have been reviewed by Mehta *et al.* (1961), Swincer *et al.* (1969) and by Greenland and Oades (1975). Appreciable yields of polysaccharide (3.5–11% of the soil organic matter) have been obtained by refluxing soils and compost with 98% methanoic (formic) acid (Parsons and Tinsley, 1961), although this procedure might be expected to lead to decarboxylation of uronic acids. However, 0.5N NaOH is probably the best of the aqueous solvents where maximum yield of polysaccharide is desired. Swincer *et al.* (1968) found that pretreatment of soil with 1N HCl or HF (but not with 0.1N H_2SO_4) significantly increased polysaccharide yield in the basic extract. When the residue was acetylated, by means of acetic anhydride and concentrated H_2SO_4 at 60 °C for 2 hours, and extracted in chloroform, the yield of carbohydrate material was increased by 23%. The amounts of carbohydrate extracted by the sequence 1N HCl, 0.5N NaOH, and the acetylated material ranged from 57 to 74% of the sugar contents of the soils.

Soil polysaccharides can be contaminated by proteins or peptides, and amino acid residues are often reported in hydrolysate digests (e.g. Finch *et al.*, 1968; Swincer *et al.*, 1968). Deproteination can be achieved by the Sevag method

(Staub, 1965) as applied by Bernier (1958) and Mortensen (1960) or by trifluorotrichloroethane (Markowitz and Lange, 1965).

Removal of brown humic materials is a major problem in the purification of soil polysaccharides. There is evidence to indicate that these are bound to each other by hydrogen bonding, and by covalent bonds such as the phenolic glycoside linkage. The best methods so far applied for the removal of humic substances are gel chromatography (Hayes *et al.*, 1975b; Barker *et al.*, 1965, 1967), ion-exchange techniques and adsorption on polyclar-AT (a polyvinyl pyrrolidone product) or nylon-coated celite (Swincer *et al.*, 1968). Salts and low molecular weight materials can also be removed by gel chromatography by using a gel with a very low exclusion limit (see Section 3.5.2).

Fractionation of soil polysaccharides. Polysaccharides isolated from soil will contain a mixture of components of varying molecular weight, charge density, and monosaccharide composition. The use of paper and column electrophoresis techniques (Clapp, 1957) for fractionation of polysaccharides has been superceded by gel chromatography (Granath, 1965; Lee and Montgomery, 1965; Churms, 1970) for fractionations on the basis of molecular weight, and anion-exchange chromatography (Neukom and Kuendig, 1965) for separations on the basis of charge density. For gel column-chromatography dilute salt solutions should be used as eluents in order to suppress possible repulsion effects between the polysaccharide and residual charges on the gel. Buffered salt gradients are generally used as eluents in ion-exchange chromatography experiments. This allows the neutral components to emerge first, and the increasingly charged materials are eluted as the salt concentration in the gradient is increased (Barker *et al.*, 1967; Finch *et al.*, 1967, 1968; Hayes *et al.*, 1975b).

Sodium tetraborate complexes to varying extents with sugars (depending on the *cis–trans* relationships of their hydroxyls) to confer different charge characteristics to them. Clapp (1957) made use of this principle to isolate a neutral polysaccharide and, by column electrophoresis, to isolate and identify the monosaccharides in the hydrolysate digest. (Later Clapp and Davis, 1970, quantitatively estimated the sugars in a *Rhizobium* polysaccharide hydrolysate by this technique.) Similar types of complexes can be formed with the component sugars in polysaccharides, and these confer different charge characteristics to otherwise neutral polymer structures. Finch *et al.* (1967) have made use of this principle to check the homogeneity of neutral polysaccharides isolated by gel filtration and anion-exchange chromatography techniques. One polysaccharide, isolated from material precipitated by sodium bicarbonate, had a molecular weight (ultracentrifuge) of *ca.* 50,000, and moved as a single component during free boundary electrophoresis in borate (pH 9.1), phosphate (pH 7.0), and barbiturate (pH 9.0) buffers. The other neutral component stayed in solution in the bicarbonate medium, and moved as a single component during electrophoresis in phosphate and barbiturate buffers. In borate buffer, however, it

Table 3.6 Sugar components of soil polysaccharides and of soil polysaccharide fractions.

Reference	OM (%)	Fraction and MW data	Sugar composition (%)										
			Gl	Gal	Man	Xyl	Arab	Rib	Fuc	Rham	UA	AS	Others
Parsons and Tinsley (1961)													
Sandy loam	3.5	—	33.6	19.1	18.0	7.9	7.9	—	—	13.5	15.8	5.2	—
Meadow		—	37.7	20.1	18.2	8.2	7.6	—	—	8.2	17.9	5.0	—
Peat		—	26.8	20.8	15.8	8.9	7.9	—	—	18.8	16.0	3.3	—
Podzol A_o		—	33.6	23.1	17.9	7.5	6.7	—	—	11.2	16.3	3.1	—
Podzol B_h		—	31.8	18.2	20.5	9.1	6.8	—	—	13.6	13.5	4.3	—
Compost 1[a]		—	42.3	15.4	15.4	15.4	11.5	—	—	—	8.4	5.0	—
Compost 2[a]		—	47.2	16.7	13.9	13.9	8.3	—	—	—	6.7	4.3	—
Swincer *et al.* (1968)													
Old pasture	4.1	(HCl) 4×10^3–10^5 MW	28.1	21.0	19.4	5.1	7.7	0.4	7.8	10.5			
		(HCl) $>10^5$	31.5	15.5	20.8	6.5	7.1	0.5	5.5	12.5			
		(NaOH) $<4\times10^3$	27.4	11.1	26.8	11.0	12.2	4.7	7.5	9.1			
		(NaOH) 4×10^3–10^5	26.8	13.4	19.3	15.9	10.7	2.8	3.8	5.4			
		(NaOH) 10^5	32.9	15.3	17.2	11.0	6.5	0.4	7.5	9.1			
Fallow-wheat rotation	2.0	(HCl) $<4\times10^3$	63.1	9.7	7.3	3.1	12.6	1.4	1.4	1.4			
		(HCl) 4×10^3–10^5	44.4	22.1	21.7	3.0	1.7	0.1	3.3	3.7			
		(HCl) $>10^5$	30.2	10.3	22.9	10.6	5.2	0.5	8.7	11.7			
		(NaOH) $<4\times10^3$	36.6	10.1	24.7	8.3	9.0	3.4	3.4	4.1			
		4×10^3–10^5	36.5	15.2	20.9	12.6	9.2	1.1	1.7	2.8			
		$>10^5$	36.0	13.6	23.0	8.8	4.5	0.5	4.4	9.2			
Finch *et al.* (1968)													
		1	36.0	10.0	17.0	7.9	5.7	—	6.5	14.0	2.1	—	—
	80	2	60.0	6.0	11.0	6.9	2.9	—	10.2	0	3.0	—	—
		3	21.0	13.0	19.0	8.4	1.8	—	13.0	18.0	3.0	—	—
		4	22.0	17.0	6.4	4.2	13.0	—	13.0	11.0	14.0	—	—
Hayes *et al.* (1975b)													
		5	68.9	9.0	9.0	3.6	2.6	—	1.9	5.3	—	—	1.8
	80	6	32.3	21.6	8.7	18.8	10.3	—	3.3	4.4	—	—	1.0
		7	70.8	5.9	5.5	8.1	5.0	—	1.9	2.3	—	—	1.0
		8	19.5	31.8	17.3	7.9	4.0	1.5	3.5	12.7	—	—	1.5
		9	18.1	33.2	16.7	8.9	4.7	0.7	5.2	11.5	—	—	0.9
		10	21.3	24.3	16.9	11.2	8.3	0.6	7.5	9.1	—	—	1.0

Gl = glucose; Gal = galactose; Man = mannose; Xyl = xylose; Arab = arabinose; Rib = ribose; Fuc = fucose; Rham = rhamnose; UA = uronic acids; AS = amino sugars; HCl and NaOH refer to soil extractants; MW = molecular weight values estimated from gel filtration data; Fractions 1–10 were obtained by gel filtration and anion-exchange chromatography.

[a] Compost samples were taken after 11 and 48 months, respectively.

separated into four negatively charged peaks, and this was conclusive evidence for inhomogeneity.

The isolation of a pure soil polysaccharide is still a long and tedious process despite the advantages of modern laboratory technology. However, the task must not in any way be regarded as impossible. In fact some of the polymers already isolated could be regarded as sufficiently pure to justify the initiation of preliminary structural studies on them. Further purification improvements might be made by use of additional techniques employed in polysaccharide work, such as fractional precipitation, using quaternary ammonium salts (Scott, 1965) or barium hydroxide (Meier, 1965), prior to applications of ion-exchange and gel-chromatography. Also the use of borate buffers for anion-exchange chromatography would appear to offer a valuable technique for refined final stage fractionations. Considerations might also be given to the applications of immunological procedures whereby pure polysaccharides can be isolated from antigen–antibody precipitin-reaction products.

The composition of soil polysaccharides

Cheshire (1977), when providing data for the contents of sugars in the hydrolysate of a typical arable Scottish soil (5.6 % C), listed glucose, galactose, mannose, xylose, arabinose, fucose, rhamnose, glucuronic acid, galacturonic acid, glucosamine, and galactosamine as the digest monosaccharides. In addition to these, ribose is sometimes a hydrolysis product, although it invariably appears to be present in the lowest abundance. It would appear, from research reports for widely different soil types from different parts of the world, that the same sugars are present in all soils which contain organic matter.

Table 3.6 lists sugar contents of polysaccharide materials isolated from some British and Australian soils. The data are not complete in any instance. However those in the paper by Swincer *et al.* (1968), for the two Urrbrae fine sandy loam samples, indicate that amino acids and amino sugars amounted to 20–100 % and 10–15 % of the sum of the weights of the neutral sugars, respectively. The uronic acid contents for samples with molecular weights greater than 4000 were 30–40 % as large as the neutral sugar contents. These polysaccharides were extracted with HCl (1N) or NaOH (0.5N) solutions, and the data are quoted for fractions isolated by gel chromatography techniques.

Finch *et al.* (1968) estimated that amino acid contamination amounted only to 1–15 % of the sugar content of their polysaccharides isolated from the precipitate formed when the 0.6N H_2SO_4 soil extract was neutralized with sodium bicarbonate. The four polysaccharides in Table 3.6 were isolated by the anion-exchange chromatography of materials which were eluted as a reasonably discrete band during gel chromatography.

The six fractions described by Hayes *et al.* (1975b) were also obtained by anion exchange chromatography followed by treatment with Sephadex of samples which had previously been fractionated by gel chromatography on

Biogel. Extraction was with ethylenediamine and the polysaccharides were contained in the fulvic acid materials.

Parsons and Tinsley (1961) extracted their polysaccharides with anhydrous formic acid containing LiBr, and the polysaccharides were precipitated with cetavalon (a quaternary cetyl ammonium salt). This work was carried out before anion-exchange and gel chromatography techniques were used in soil polysaccharide studies. All of the Parsons and Tinsley polysaccharide materials were treated in the same way, and it can be seen that the sugar contents of the different soil extracts were quite similar. The plant origins of the composts are indicated by the relatively high glucose, xylose, and arabinose contents.

The polysaccharides of Swincer *et al.* (1968) were fractionated on the basis of molecular weight differences. It is clear from their data that the composition of the polysaccharides were influenced by cultivation practices. Differences between the compositions of the different molecular weight fractions and the influences of the extractants were most striking in the cases of samples from the cultivated soil. By contrast the various samples from the old pasture soil were very similar.

The data of Finch *et al.* (1968) and of Hayes *et al.* (1975b) as shown in Table 3.6, highlight the fact that the similarity in the molecular weights of soil polysaccharide extracts cannot be regarded as a criterion for homogeneity. Some of the polysaccharides of similar molecular sizes, which were fractionated by ion-exchange chromatography, were distinctly different from each other, although there were similarities between others. The high glucose contents of fractions 1, 6, and 10 might suggest plant origins, but this was not substantiated by the relative abundances of xylose and arabinose.

Where only small amounts of neutral sugars are present in the digests of extensively fractionated soil polysaccharides it is tempting to infer that these arise from contaminations by other polysaccharides. However, it will be seen from an examination of the relative abundances of the sugars in all of the hydrolysates in Table 3.6 that it is unlikely that there are fewer than four sugar residues in the majority of soil polysaccharides.

3.6.4 Persistence of Polysaccharides in Soil

Reese (1968) and Finch *et al.* (1971) have discussed some of the features of enzymatic transformations which may be involved in the degradation of soil polysaccharides. We will only briefly refer here to some of the concepts which are involved in these degradations.

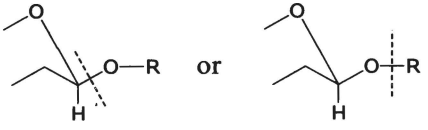
Polysaccharide biosynthesis and degradation is unified by the glycosyl transfer reaction which can be expressed by

$$\underset{\text{(donor)}}{\text{G—OR}} + \underset{\text{(acceptor)}}{\text{R'O—H}} \longrightarrow \text{G—OR'} + \text{RO—H} \qquad (3.62)$$

In the biosynthesis reaction the acceptor is the growing polysaccharide chain, R′OH, and in degradation the acceptor is a small molecule such as water. The glycosyl group G is transferred in each case. Thus the degradation reaction may be represented as

$$G_1—O—G_2 + HOH \longrightarrow G_1OH + HOG_2 \tag{3.63}$$

Cleavage can take place as indicated by the broken lines in (a) and (b) in scheme (3.64), but almost invariably glycone–oxygen fission (a) occurs.



(a) (b) (3.64)

Two classes of degradative enzymes exist; one class (exoenzymes) catalyses the cleavage of the non-reducing terminal residues, and the other (endo-enzymes) that of glycosidic bonds throughout the polymer chains. It is easy to distinguish between these two classes because molecular weight is more readily decreased by the action of exoenzymes. However, their action can be blocked by the presence of bulky substituents, such as *O*-acetyl groups on the sugar hydroxyls, which inhibit the approach of the enzymes to the glycosidic bonds. In many cases minor modifications in the donor glycone, such as inversion of configuration at one carbon atom leads to greatly reduced activity. However, the influence of the different types of linkages is most important in so far as donor aglycone specificity in hydrolysis is concerned. Thus branching, and changes from $1 \rightarrow 4$ to $1 \rightarrow 6$ linkages, as illustrated in Figure 3.20 will give rise to different stereochemical arrangements in the vicinity of the glycosidic linkages, and will require different enzyme systems for cleavage. It should also be remembered that all glycosidases are specific for the α or β configuration at the anomeric carbon atom.

We concluded in Section 3.6.2 that it is highly likely that most of the polysaccharides synthesized in soil contain four or more sugar residues. The greater the variety of residues and of linkages the greater should be the resistance of the polysaccharide to enzymatic attack. Unfortunately lack of structural information at this time does not allow predictions to be made on the basis of these criteria. However, it is highly likely that a variety of enzymes are required in order to achieve extensive depolymerization. Cheshire and Anderson (1975) have shown that single purified enzymes have little degradative influence on soil polysaccharides and this is understandable in the light of the foregoing considerations.

Finch *et al.* (1971) have referred to unpublished results which indicated that polysaccharide mixtures from an organic soil were less susceptible to decomposition (as measured by CO_2 evaluation) than glucose, sucrose, cellulose, and

ryegrass when each of these different materials was supplied as the sole source of carbon for microorganisms in aerated liquid culture media. Some of the polysaccharides decompose quite readily, as was shown by Cheshire *et al.* (1974) when a large proportion of their alkali-soluble soil polysaccharide was decomposed in 4–8 weeks during incubations with fresh soil suspensions.

It would appear, however, that soil polysaccharides are protected to some extent in their native environments. Among the reasons offered for this are (Cheshire, 1977): (i) adsorption by clays resulting in a degree of inaccessibility to enzymes and microorganisms; (ii) a preservative effect from tannins; and (iii) the formation of complexes with metals.

Lynch and Cotnoir (1956) have provided evidence to show that adsorption on montmorillonite slows microbial degradation of polysaccharides, and Finch *et al.* (1967) and others have observed interlamellar adsorption of soil polysaccharide by Na^+-montmorillonite. Such intimate associations with the clays would alter the conformations of the molecules and inhibit the approach of the hydrolytic enzymes to the glycosidic linkages. Chemical degradation is also slowed as the result of adsorption, and Finch *et al.* (1967) have shown that uptake of periodate by a kaolinite adsorbed soil polysaccharide was ten times slower than that by the polysaccharide in solution. Arguments along these lines might also apply for polysaccharides adsorbed to humic substances or bound to them by Ca^{2+} or Mg^{2+} bridging (Barker *et al.*, 1967), or entrapped within coiled humic acid molecules.

The preservative effects of tannins, as reviewed by Cheshire (1977) might be regarded as an extension of this argument. Tannins have some of the structural features of humic substances and could form covalent (e.g. phenolic glycosides), ionic, and hydrogen bonded linkages with the polysaccharide.

Martin *et al.* (1966) proved that the resistance to decomposition of some polysaccharides in the soil is increased by complexing with Fe, Cu, and Zn. This might be related to inhibition of hydrolytic enzymes and/or to changes in polymer conformations resulting from bridging by the metals of the carboxyl groups of the uronic acid residues.

3.6.5 Soil Polysaccharide–Clay Interactions

The beneficial effects of polysaccharides on soil structure can almost certainly be attributed to their interactions with clays. Some emphasis is given to the mechanisms of polymer adsorption by clays in the chapter on 'Adsorption' by Burchill, Greenland, and Hayes in the companion volume of this book, and more detailed attention is given there to clay–polysaccharide interactions. Here we will be concerned in a more general way with the adsorption of polysaccharides of known structures, and of soil polysaccharides with reasonably well-defined clay preparations.

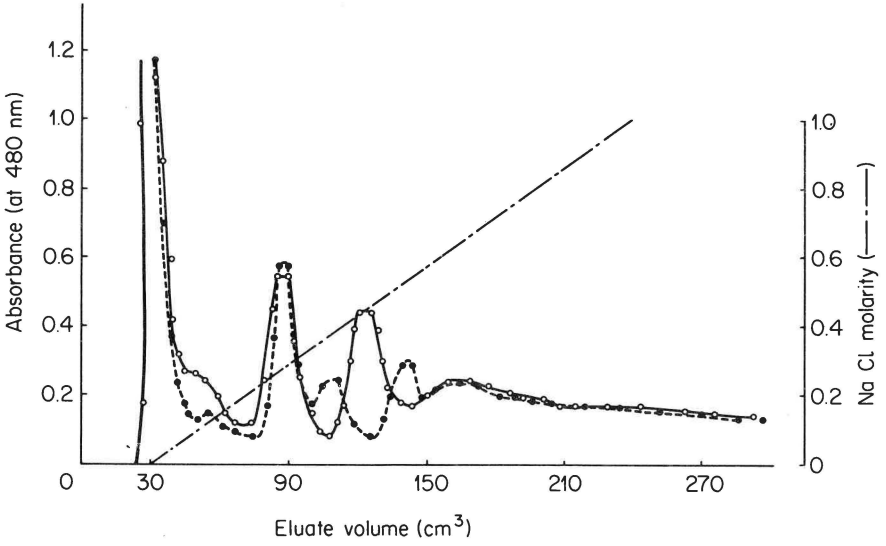


Figure 3.22 Anion-exchange chromatography on DEAE-A50 Sephadex in *p*H 6.0 phosphate buffer of a soil polysaccharide mixture before (—o—o—) and after (—•—•—) treatment with H^+-montmorillonite. (After Finch *et al.*, 1967)

Clapp and his associates (Clapp *et al.*, 1968; Clapp and Emerson, 1972; Olness and Clapp, 1973, 1975) have carried out a number of studies on the adsorption by montmorillonite of a variety of microbial polysaccharides whose structures are understood. These included an extracellular rhizobial polysaccharide and two dextrans, B-512F, and a commercial product 'Polytran' with molecular weights of the order of 2×10^6. The dextrans differed in the nature of the linkages of the glucose units; B-512F (signifying the NRRL code for the synthesizing strain of *Leuconastoc mesenteroides*) and 'Polytran' were linked 95 % α-1 → 6, 5 % α-1 → 3 and 75 % β-1 → 3, 25 % β-1 → 6, respectively.

Olness and Clapp (1973, 1975) have clearly shown that the nature of these linkages significantly affected the amounts (and hence the conformations) of polymers adsorbed on the clay surfaces. In the case of B-512F almost all of the adsorbed dextran could lie on the Na^+-montmorillonite surface. Polytran, however adsorbed significantly in excess of the amount necessary to cover the adsorbent surface and this would suggest the presence of loop and train conformations. The authors concluded that the availability of the primary alcohol groups (because of the lesser involvement of the C-6 hydroxyl in linkages) in Polytran enabled this polymer to form a stable complex with the clay from a shorter length of polymer chain than for B-512F.

It will not be possible to make comparable predictions about the mechanisms of soil polysaccharide–clay interactions until more is known about the polymer structures. However, there is ample evidence from different laboratories to

prove that at least some soil polysaccharides are adsorbed by clays. Data from Finch *et al.* (1967), summarized in Figure 3.22, show that all of the polysaccharides which they isolated were not adsorbed to the same extents by a resin treated H^+-montmorillonite clay preparation. Comparison of the elution patterns of polymer from DEAE-A50 anion-exchange Sephadex before and after reaction with the clay shows that most of the component eluted at *ca.* 120 cm^3 (in a sodium chloride gradient in pH 6.0 phosphate buffer) was adsorbed by the clay. Other work by Barker *et al.* (1967) suggested that this component contained one uronic acid unit per 6 to 7 sugar residues. More highly charged components (eluted at 130–250 cm^3) were not adsorbed. Only a small proportion of the charge on these components was, however, contributed by uronic acids. Very little adsorption was observed for the essentially neutral (eluted at 25–40 cm^3) and lesser charged (75–100 cm^3) polysaccharide components.

The study by Lynch *et al.* (1956) of the adsorption of cellulose preparations, starch dextrins, glycogen, and corn polysaccharides by H^+- and Ca^{2+}-clay preparations showed that these polymers were adsorbed to different extents. Carboxymethylcellulose ($\alpha\,\beta$-1 $\rightarrow$ 4 linked polyglucose structure in which the —OH substituent on C-6 of glucose is substituted by —COOH) was the least adsorbed of the cellulose derivatives by H^+-montmorillonite. Also only small amounts of alginates and pectinates (charged polysaccharides) were adsorbed by H^+- and Na^+-montmorillonite. At the low pH values of the H^+-clay medium dissociation of the uronic acid carboxyls would not be expected, and some hydrogen bonding (as suggested from infrared evidence by Lynch *et al.*, 1956) between the clays and acidic polysaccharides might be predicted. But the failure of the highly acidic polysaccharides to be extensively adsorbed would suggest that hydrogen bonding was inhibited in these instances.

It is not necessary, however, to invoke specific mechanisms such as hydrogen bonding for polymer adsorptions by clays. As Greenland (1965) and Parfitt and Greenland (1970) have stated, multiple dispersion forces, arising from the numerous points of contact per polymer segment, as well as entropy factors may be most important. The increased entropy which would favour the reaction could be provided by displacement of a number of small molecules, such as water, by a single macromolecule.

Therefore it would appear that the ability of any (soluble) polysaccharide to adsorb on clay may be governed by the stereochemistry of the polymer because this can determine the extent to which the macromolecules can make contacts with the surfaces. Also the conformations of the polymers in the adsorbed states may well determine whether or not a particular soil polysaccharide will stabilize aggregates. For instance part of the contribution to aggregate stabilisation might arise from polysaccharide loops from one adsorbing surface forming trains on another (see Chapter by Burchill, Greenland and Hayes in the companion volume). However, such proposals must be regarded as speculative until results from further adsorption studies are available.

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